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## CHLOROPHYLL D, A GREEN PIGMENT OF RED ALGAE

By WINSTON M. MANNING AND HAROLD H. STRAIN

(From the Carnegie Institution of Washington, Division of Plant Biology, Stanford University, California)

(Received for publication, July 1, 1943)

Recent investigations have emphasized the diversity of chlorophylls in plants (1-3). Higher plants and green algae contain chlorophyll *a* and chlorophyll *b*. By contrast, diatoms, dinoflagellates, and brown algae do not contain chlorophyll *b* but do contain, in addition to chlorophyll *a*, a characteristic green pigment, chlorophyll *c* (1, 2).

Red algae (Rhodophyta) are sharply distinguished from all other plants with respect to anatomy, life history, and the occurrence of certain proteinaceous pigments. In order to determine whether or not this distinctiveness is reflected by the occurrence of unique green pigments, we have investigated the chlorophylls of several marine species of red algae.

Earlier work has shown that chlorophyll *a* is the principal chlorophyll of red algae, and that chlorophyll *b* is absent, or present only in traces (4-7). The occurrence of chlorofucine (chlorophyll *c*) in red algae, reported 70 years ago by Sorby (4), has not been verified (2, 5, 7). Until now, no other chlorophyll-like pigment has been reported for red algae.

### EXPERIMENTAL

#### *Material and Methods*

The red algae were collected at low tide near Moss Beach north of Half Moon Bay, California. We are indebted to Dr. Gilbert M. Smith of Stanford University for identification of these algae. In most cases the material was used within 24 hours after collection. When not used within this time, the algae were moistened with sea water and stored at 6° in a loosely covered container.

The methods employed for extraction and purification of the pigments, and for measurement of spectral absorption, were essentially those described in preceding papers (1, 2). When modification or extension of these methods was necessary, the procedures are described in the sections pertaining to the preparation of individual compounds.

For most species of red algae, chlorophyll extraction required several hours when the fresh thalli were treated directly with methanol. For one species, *Erythrophyllum delesserioides*, extraction with methanol was nearly complete in 20 to 30 minutes. When the algae were killed by



immersion for 1 minute in boiling water, subsequent extraction of chlorophylls with methanol was much more rapid, being complete in from 10 to 30 minutes for all species. Extraction of the chlorophylls with ethanol, with pyridine, or with acetone-water (4:1 by volume) was less rapid than with methanol.

None of these solvents extracted appreciable quantities of phycoerythrin, the characteristic red pigment of red algae (8, 9). After exhaustive extraction with methanol, the algal material was always bright red or pink, even for species which were originally green, brown, or purple. For the inexperienced collector, the residual red color, which appears within a few minutes upon extraction of the heat-killed organism, affords a simple and rapid means of recognizing as red algae (Rhodophyta) certain species which otherwise might be mistaken for brown or green algae.

Spectral absorption and fluorescence measurements of extracted pigments were made with a photoelectric spectrophotometer, constructed by Smith (10). Most of the absorption spectra shown in this paper are plotted as  $\log \log (I_0/I)$  versus wave-length. The lability of the pigments and the presence of colorless concomitants precluded determination of the specific absorption coefficients

### Results

*Natural Occurrence of Chlorophyll d*—In Fig. 1 are presented the characteristic spectral absorption values for a methanol extract of *Erythrophyllum delesserioides*, killed by immersion for 1 minute in boiling water. The curve for chlorophyll *a* (1) is superposed arbitrarily on the absorption values for the extract. Coincidence of the circles with the curve in the regions of maximum absorption indicates that the extract contains a large proportion of chlorophyll *a*. Between 680 and 720  $m\mu$ , absorption values for the extract diverge a great deal from the chlorophyll *a* curve. This divergence demonstrates the presence of another pigment (or pigments) that absorbs strongly at the longer wave-lengths. The relative spectral absorption by this second pigment in the extracts of *Erythrophyllum* was estimated by subtraction of the fractional absorption due to chlorophyll *a* from the total absorption, as indicated by the formula in the caption for Fig. 1. The values obtained in this way, shown in Fig. 1, correspond with the characteristic absorption curve for a green pigment prepared by chromatographic adsorption from red algae (see Fig. 3). Superposability of the absorption curve on the difference values indicates that the absorption in the region 680 to 720  $m\mu$ , over and above that due to chlorophyll *a*, is primarily or solely due to this second green pigment. For this pigment, we propose the name *chlorophyll d*.

If chlorophyll *d* is a natural constituent of red algae, in the same sense

that chlorophyll *a* is a natural constituent, the amount of this pigment relative to chlorophyll *a* should not vary when the plants are killed in different ways and the chlorophylls are extracted completely. Of the species available for such a test, *Erythrophyllum delesserioides* was most suitable because of the relatively high chlorophyll *d* content and rapid extractability of the pigments.

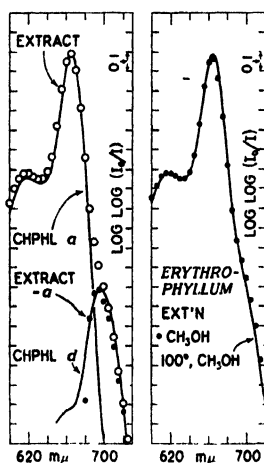


FIG. 1

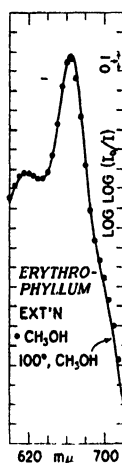


FIG. 2

FIG. 1. Absorption values for pigments in a methanol extract of *Erythrophyllum delesserioides*. The circles represent absorption by all pigments in the extract. The chlorophyll *a* curve (line) represents absorption by chlorophyll *a* in the extract. The dots represent the calculated absorption values for the difference between the absorption by the extract (circles) and the absorption by chlorophyll *a* in the extract. These difference values are calculated as  $\log_{10} [(\log_{10} (I_0/I) \text{ for extract}) - (\log_{10} (I_0/I) \text{ for chlorophyll } a \text{ in extract})]$ . The curve for chlorophyll *d* (line) is from Fig. 3 and is arbitrarily superimposed on the difference value at 695  $m\mu$ .

FIG. 2. Absorption curves for pigments in methanol extracts of *Erythrophyllum delesserioides*. The line is for an extract of plant material which had been killed by immersion for a minute in boiling water. The dots represent absorption by an extract of fresh material. The curves are arbitrarily superposed at 650  $m\mu$ .

Fig. 2 shows that the absorption curve for a methanol extract of fresh *Erythrophyllum* (not heat-treated) is in fair agreement with the curve for an extract of heat-killed material. The agreement indicates that the amount of chlorophyll *d* relative to chlorophyll *a* was not appreciably altered by the variation in treatment.

For methanol extracts, the ratio of the absorption density at 665  $m\mu$  (chlorophyll *a* maximum) to the density at 700  $m\mu$  (near the chlorophyll *d* maximum) is a sensitive test for the presence of chlorophyll *d* (den-

sity =  $\log_{10} (I_0/I)$ ). For pure chlorophyll *a* in methanol, this ratio is approximately 90. With increasing amounts of chlorophyll *d*, the ratio rapidly decreases until for pure *d* it is 0.25. The numerical values of this ratio for methanol extracts of several species of red algae are shown in Table I. The presence, even in small amounts, of light-scattering material or of various impurities, would appreciably lower the observed values for the ratio; so that values in Table I greater than 45 or 50 cannot be regarded as conclusive evidence for the presence of chlorophyll *d*. Moreover, pigment extraction was incomplete for some of the species listed in Table I; hence, these values should be regarded as only approximate indications of the relative amounts of chlorophylls *a* and *d*.

TABLE I

Ratios of Absorption Density at 665  $M\mu$  to Absorption Density at 700  $M\mu$  for Methanol Solutions of Pigments Extracted from Red Algae

Density =  $\log_{10} (I_0/I)$

Species	Ratio	Species	Ratio
<i>Erythrophyllum delesserioides</i>	15	<i>Plocamium pacificum</i> ..	46
<i>Rhodomela larix</i>	28	<i>Prionotis lanceolata</i> .....	49
<i>Endocladia muricata</i>	32	<i>Callithamnion pikeanum</i> ....	55
<i>Botryoglossum farlowianum</i>	36	<i>Microcladia coulteri</i> .	55
<i>Cryptopleura violacea</i>	36	<i>Gigartina californica</i> ..	59
<i>Gigartina papillata</i>	36	<i>Halosaccion glandiforme</i>	62
<i>Calliarthron setchelliae</i>	38	<i>Hymenena flabelligera</i>	63
<i>Iridophycus flaccidum</i>	39	<i>Odonthalia floccosa</i> .	65
<i>Gelidium purpurascens</i>	42	Chlorophyll <i>d</i> .	0.25
<i>Gigartina agardhii</i>	42	" <i>a</i>	90

*Differential Extractability of Chlorophylls a and d*—Extraction of certain fresh, unheated algae with methanol removed chlorophyll *d* more rapidly than chlorophyll *a*. As a result, the pigment mixtures removed by brief extraction contained a much higher proportion of chlorophyll *d* than did those obtained by longer, more nearly complete extraction. For example, a 5 gm. sample of *Endocladia muricata*, after extraction for 2 minutes with 100 ml. of methanol, yielded a solution with an absorption ratio (665  $m\mu$ : 700  $m\mu$ ) of 21; after extraction for 1 hour the solution contained over 3 times as much total pigment but exhibited an absorption ratio of 32, indicating more chlorophyll *a* and therefore relatively less chlorophyll *d*. In a similar experiment with *Gigartina agardhii*, extraction for 2 minutes yielded a solution very rich in chlorophyll *d*, with a ratio of only 9.8, whereas extraction for 5.5 hours yielded a solution with nearly 10 times the total pigment concentration, but with much more chlorophyll *a* in pro-

portion to chlorophyll *d*, as shown by an absorption ratio of 38. Another sample from the same collection of *Gigartina*, placed in boiling water for 1 minute, was then extracted quickly and completely, yielding a solution with an absorption ratio of 46. By contrast, the closely related species *Gigartina papillata* showed no evidence of differential extractability, extraction being equally slow for chlorophylls *a* and *d*.

Differential extraction of chlorophyll is not confined to red algae, having been observed at low temperature for chlorophylls *a* and *c* in the diatom *Isthmia nervosa* (2). In this case, chlorophyll *c* was extracted more rapidly than chlorophyll *a*.

*Separation and Properties of Chlorophyll d*—The remarkable differential extractability of chlorophylls *a* and *d* from *Gigartina agardhii* facilitated the preparation of chlorophyll *d*. Rapidly prepared extracts of this alga were not only rich in chlorophyll *d* but they also yielded pigment mixtures that were more readily separable on the adsorption columns than were mixtures obtained by complete extraction of all the green pigments. Moreover, *G. agardhii* was readily available at all seasons, high in the intertidal zone, and it remained in good condition for nearly 2 weeks when moistened with sea water and stored at 6°. *G. agardhii* was therefore used for all large scale preparations of chlorophyll *d*.

The following procedure was used in a typical preparation. Approximately 1 kilo of fresh, slightly moist *Gigartina* was chopped in a wooden bowl with a sharp knife until the pieces were reduced in size to 2 or 3 mm. This chopped material was placed in a large container with 2 liters of methanol, and agitated frequently. After 30 minutes, the green extract was poured off and the residue steeped with another liter of methanol for 5 to 10 minutes. These two methanol extracts, which contained nearly all the chlorophyll *d* but not over 15 per cent of the chlorophyll *a* originally present in the plant material, were combined in a 6 liter separatory funnel. Approximately 300 ml. of petroleum ether (b.p. < 75°) were thoroughly mixed with the methanol solution. About 500 ml. of 10 per cent sodium chloride solution were then added. The aqueous alcohol layer was separated and reextracted with 200 ml. of petroleum ether. The two petroleum ether extracts were combined, washed at least three times with water, and then passed through an adsorption column of dry powdered sugar (1),  $6.7 \times 20$  cm. The column was washed with a small amount of petroleum ether, then with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline until most of the chlorophyll *a* was carried into the percolate. At this stage, the bands on the column were not separated completely from one another. A diffuse green zone near the bottom of the column contained the chlorophyll *d*. Trailing portions of chlorophyll *a* contaminated the chlorophyll *d* band. Yellow pigments

were adsorbed below, in, and sometimes above the chlorophyll *d* band. Still higher on the column, there usually occurred a diffuse green zone which contained small amounts of chlorophyll *b*. This pigment probably came principally from traces of *Ulva*, a green alga which frequently grows attached to *Gigartina*.

For further purification of chlorophyll *d* the green zone containing this pigment was removed from the column and extracted with a mixture of petroleum ether and ethanol. (If necessary, the elutriate containing ethanol may be stored overnight.) The elutriate was washed several times with water. The remaining petroleum ether solution was then concentrated at 20°, at reduced pressure, to about 40 ml. Sometimes a precipitate formed at this stage, in which case a few ml. of alcohol-free ethyl ether were added. The pigments were then adsorbed on a short column of sugar,  $4.7 \times 8$  cm., and washed with petroleum ether until a narrow yellow zone was carried below and just free of the narrow green layer at the top of the column. This green layer was transferred to a flask. Approximately 10 ml. of methanol were added, then 75 ml. of petroleum ether, and finally 25 or more ml. of water. The contents of the flask were transferred to a small separatory funnel, the aqueous alcohol layer was removed, and the petroleum ether layer was washed three times with water. This petroleum ether solution was then filtered through another column of powdered sugar,  $4.7 \times 18$  cm. The adsorbed pigments were washed with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline. Slight remaining traces of chlorophyll *a* preceded chlorophyll *d* on the column; all residual portions of yellow pigments were adsorbed above the *d* band. When the chlorophyll *d* had been carried approximately two-thirds of the way through the column, the middle portion of the *d* band was removed, both the leading and the trailing portions being discarded. The adsorbed pigment was eluted with ether, or with methanol and petroleum ether. The resulting solution was evaporated at 20°, at reduced pressure, to a small volume. Several ml. of methanol were added, the residual ether was evaporated as before, and additional methanol was then added. The entire procedure required at least 7 or 8 hours.

Methanol solutions of chlorophyll *d* prepared in this manner are pure green, the color being intermediate between that of methanol solutions of chlorophyll *a* and chlorophyll *b*. The characteristic absorption spectrum of freshly prepared chlorophyll *d* in methanol is shown in Fig. 3. The absorption spectra of similar preparations of chlorophyll *d* from *Gigartina papillata* and from *Erythrophyllum delesserioides* agreed well with that shown in Fig. 3.

Chlorophyll *d* dissolved in u. s. p. ether had principal absorption maxima at 686  $m\mu$  and 445  $m\mu$ , with absorption approaching another maximum

at or below  $395\text{ m}\mu$ , the limit of the spectrophotometer. The chlorophyll *d* absorption maxima in ether were more pronounced than the maxima in methanol, an effect also observed with chlorophylls *a*, *b*, and *c* (1). For chlorophyll *d* in petroleum ether, the absorption maximum in the red region of the spectrum was at  $684\text{ m}\mu$ .

The wave-length of the principal absorption maximum of chlorophyll *d* could not be determined accurately with a visual spectrophotometer because of the low and rapidly changing sensitivity of the eye to light of long wave-lengths.

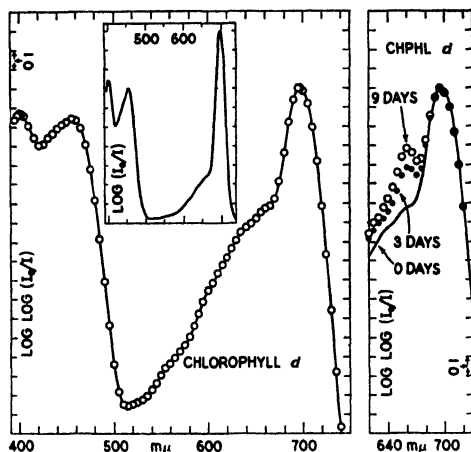


FIG. 3

FIG. 4

FIG. 3. Absorption curve for chlorophyll *d* from *Gigartina agardhii*. The inset shows the same data plotted as  $\log_{10} (I_0/I)$ . Solvent, methanol.

FIG. 4. Alteration of chlorophyll *d*. The line represents absorption by a fresh preparation of chlorophyll *d* in methanol. The dots and circles represent absorption by the preparation after standing for 3 and 9 days, respectively, at room temperature in the dark in sealed, evacuated tubes. The curves are arbitrarily superposed at  $695\text{ m}\mu$ .

Solutions of chlorophyll *d* in ether showed a deep red fluorescence with a maximum at  $693\text{ m}\mu$ . There appeared to be a diffuse secondary fluorescence maximum at about  $750\text{ m}\mu$ .

Chlorophyll *d* was readily soluble in petroleum ether, and yielded a yellow-green solution. In its relative solubility in petroleum ether as compared to methanol, chlorophyll *d* resembled chlorophyll *a* rather than the alcohol-soluble chlorophyll *c*. In adsorbability, chlorophyll *d* was intermediate between chlorophylls *b* and *b'* (11) when adsorbed on powdered sugar and washed with petroleum ether-propanol mixtures.

*Absence of Chlorophylls b and c in Red Algae*—Since small amounts of

chlorophyll *b* (probably from contamination by the green alga, *Ulva*) were often observed on adsorption columns during large scale preparations of chlorophyll *d* from *Gigartina agardhii*, a more critical test was made for the presence of chlorophyll *b* in *Gigartina agardhii* and in *Hymenena flabelligera*.

A 10 gm. sample of *Gigartina agardhii* was carefully selected on the basis of freedom from appreciable contamination by other algae, then killed by heating at 100° for 1 minute. The pigments were extracted with methanol and an aliquot was removed for spectrophotometric determination of the amount of chlorophyll *a*. The pigments in the remaining solution were quantitatively transferred to petroleum ether and adsorbed and washed on a column of powdered sugar (3 × 20 cm.). No band of chlorophyll *b* was observed on the column. Three contiguous zones of the column, including the entire region where traces of chlorophyll *b* could have been adsorbed, were removed. The pigments from each zone were eluted separately, dissolved in methanol, and examined spectrophotometrically. Small amounts of alteration products of chlorophyll *a* (2) were observed in these zones but there was no positive evidence for the presence of chlorophyll *b*. At most, the amount of chlorophyll *b* could not have exceeded 0.3 per cent of the amount of chlorophyll *a* present in the original extract. A nearly identical procedure was used for *Hymenena flabelligera*, with similar results.

Chlorophyll *c* was not found by spectrophotometric examination of the extracts of red algae, nor by adsorption of the pigments on columns of sugar.

From these experiments it is evident that chlorophylls *b* and *c* either are absent from the red algae which were examined, or are present only in traces.

*Metal Constituent of Chlorophyll d*—On two occasions fresh preparations of chlorophyll *d*, each from 2 kilos of *Gigartina agardhii*, were transferred to petroleum ether and washed thoroughly with water to remove possible water-soluble impurities. The petroleum ether solutions were evaporated nearly to dryness, below room temperature at reduced pressure. In each case approximately 2 ml. of ether were added to the residue. The ether solutions were transferred to small weighed platinum crucibles and evaporated to dryness. The residues were then ignited at red heat for approximately 20 minutes. The residual ash was white and in each case weighed  $0.12 \pm 0.008$  mg. Water failed to dissolve the ash. A little hydrochloric acid was added and the resulting solutions were transferred to volumetric flasks and made up to 15 ml.

In order to determine whether or not the ash from chlorophyll *d* contained magnesium, the solutions were analyzed colorimetrically for

magnesium, with Titan yellow or *p*-nitrobenzeneazo- $\alpha$ -naphthol as reagents (12). With both reagents, sodium cyanide was used to reduce possible color quenching by other metals. In every case the blank, the standard, and the test solutions were made up to 10 ml. and measured spectrophotometrically (at 535  $m\mu$  for Titan yellow and at 650  $m\mu$  for *p*-nitrobenzeneazo- $\alpha$ -naphthol). Four aliquots from the solution prepared from one sample of ash were analyzed with Titan yellow and gave values of 0.115, 0.095, 0.110, and 0.102 mg., respectively (average 0.106 mg.), for the magnesium oxide content of the ash. For the other preparation of ash, three determinations with Titan yellow gave values of 0.097, 0.119, and 0.113 mg., respectively, two determinations with *p*-nitrobenzeneazo- $\alpha$ -naphthol gave values of 0.109 and 0.105 mg. The average for these last five determinations was 0.109 mg. Considering the very small quantities involved, these average values for magnesia content are in satisfactory agreement with the total weights of the ash ( $0.12 \pm 0.008$  mg. in each case).

Because of the specificity of Titan yellow, and because the two colorimetric reagents gave results agreeing with each other and also with the weights of ash, it may be concluded that the metal constituent of the chlorophyll *d* molecule is magnesium.

*Isomerization of Chlorophyll d*—Chlorophyll *d* dissolved in methanol and allowed to stand in the dark, either in the presence or absence of oxygen, undergoes alteration, with an absorption band at 661  $m\mu$  gradually becoming prominent (see Fig. 4). This change in spectrum has been found to result from isomerization of chlorophyll *d*.

In a typical experiment, a chlorophyll *d* solution, after standing for 9 days, was transferred to petroleum ether, adsorbed on sugar, and washed with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline. Instead of the original single zone, at least three or four bands were visible on the column (see Fig. 5). Topmost, except for traces of one or two strongly adsorbed pigments, was the green band of chlorophyll *d*. Below this and well separated from the *d* band were two adjacent bands incompletely separated from each other, the upper yellow-green zone merging into a lower blue-green zone. Farther down on the column was a single, pale, blue-green band.

After the pigments from the two lower bands were eluted separately and heated, or allowed to stand in methanol for a few days, each was converted into a mixture of the four pigments previously observed to arise from chlorophyll *d*. The second green band (second from the top of the column, Fig. 5) was not obtained in sufficient purity for a critical test, but probably it too was converted into a similar mixture. This behavior, as well as evidence presented in subsequent sections, indicates that these four pigments probably are interconvertible isomers. The



same series of pigments was formed by heating a methanol solution of chlorophyll *d* in a sealed evacuated tube at 80–82° for 2 hours. For the

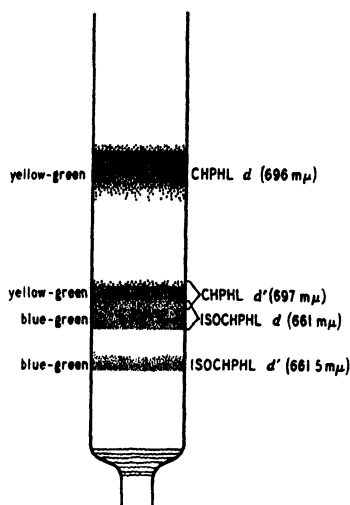


FIG. 5. Separation of chlorophyll *d* and its isomers on powdered sugar after washing with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline. The wave-length values are those of the principal absorption maxima for the pigments dissolved in methanol.

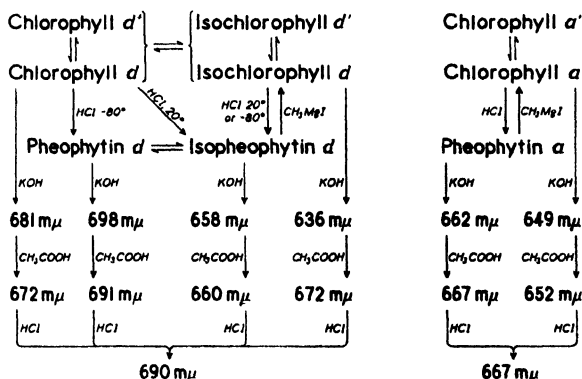


FIG. 6. Isomerization and other reactions of chlorophyll *d* and chlorophyll *a*. The wave-length values are those of the principal absorption maxima for products of alkali treatment dissolved in methanol plus potassium hydroxide, and for products of acid treatment dissolved in ether.

three isomers adsorbed in succession below chlorophyll *d* on sugar columns, we propose the names *chlorophyll d'*, *isochlorophyll d*, and *isochlorophyll d'*

(Fig. 5). The interrelations of these isomers and some of their decomposition products are shown in Fig. 6.

Because it was extremely difficult to separate chlorophyll *d'* and isochlorophyll *d* by adsorption, only an approximate absorption curve for chlorophyll *d'* was obtained (see Fig. 8). As will be described in a subsequent section, isochlorophyll *d* free from chlorophylls *d* and *d'* was obtained by addition of magnesium to one of the pheophytins obtained from chlorophyll *d*. The absorption spectrum of isochlorophyll *d* prepared in this way and dissolved in methanol, in so far as it has been determined, is shown by the circles in Fig. 7. For comparison, the spectrum of chloro-

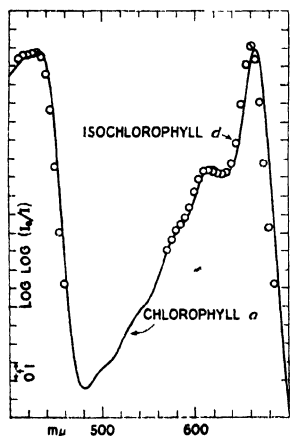


FIG. 7

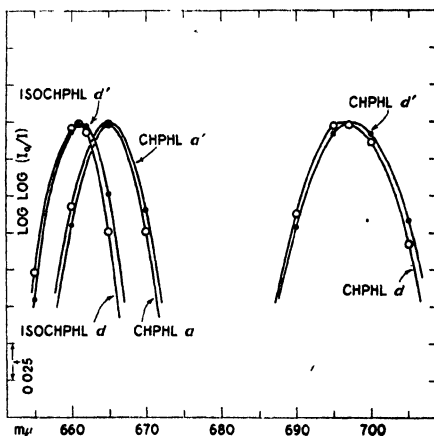


FIG. 8

FIG. 7. Absorption curves for chlorophyll *a* (line) and isochlorophyll *d* (circles). Solvent, methanol. The curves are arbitrarily superposed at 430  $m\mu$ .

FIG. 8. Absorption maxima for chlorophylls *a* and *d* and their isomers. Solvent, methanol. The curves are arbitrarily superposed. The scale is expanded to 8 times that of the absorption curves in the other figures

phyll *a* in methanol is shown by the line in Fig. 7. The spectra are remarkably similar. To determine whether the adsorbabilities were also similar, a mixture of isochlorophyll *d* and chlorophyll *a* was dissolved in petroleum ether and adsorbed on a column of powdered sugar,  $3 \times 22$  cm. When the adsorbed pigments were washed with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline, the two bands separated very slowly. When the bottom of the chlorophyll *a* band had reached a point 17 cm. below the top of the column, the bottom of the isochlorophyll *d* band was 15.5 cm. from the top of the column, with a space of only a few mm. between the two bands.

A limited number of absorption measurements indicate that the spectrum

of isochlorophyll *d'* is nearly identical with that of isochlorophyll *d*, except for a shift of about 0.5  $\mu$  toward longer wave-lengths (Fig. 8). Absorption measurements of an impure preparation of chlorophyll *d'* indicate that its spectrum bears a similar relation to the spectrum of chlorophyll *d* (Fig. 8). This figure also shows that the spectrum of chlorophyll *a'*, an isomer of chlorophyll *a* (11), is similarly related to the spectrum of chlorophyll *a*.

Chlorophyll *d* adsorbed on columns of powdered sugar was isomerized slowly. When the chlorophyll *d* band was washed with petroleum ether containing 0.5 per cent *n*-propanol, the diffuse lower portion of the band contained small quantities of isochlorophyll *d*, as indicated by spectrophotometric examination. This was the principal reason for discarding the lower portion of the chlorophyll *d* band in the course of the preparation and purification of chlorophyll *d*.

The precise equilibrium was not determined for mixtures of chlorophyll *d*, chlorophyll *d'*, isochlorophyll *d*, and isochlorophyll *d'*. Fig. 4 gives some indication of the amounts of the chlorophyll *d* and isochlorophyll *d* isomers under conditions approaching equilibrium. It was observed that interconversion of chlorophylls *d* and *d'* and of isochlorophyll *d* and isochlorophyll *d'* was more rapid than the interconversion of chlorophyll *d* and isochlorophyll *d*. Under conditions near equilibrium, the concentration of chlorophyll *d* was perhaps 4 times that of chlorophyll *d'*. A similar ratio was observed for mixtures of isochlorophyll *d* and isochlorophyll *d'*.

Because of its similarity to chlorophyll *a*, isochlorophyll *d*, even in fairly large quantities, could easily be overlooked upon spectroscopic or chromatographic examination of fresh methanol extracts of red algae. However, a careful examination of extracts and chromatograms prepared rapidly from *Gigartina agardhii* and from *Erythrophyllum delesserioides* indicated that little or no isochlorophyll *d* was present. Evidently isomerization of chlorophyll *d* to isochlorophyll *d* does not occur appreciably in the living organisms.

*Pheophytins from Chlorophyll d and Isochlorophyll d*—Depending upon the treatment, chlorophyll *d* yields either or both of two interconvertible pheophytins (see Fig. 6). For preparation of the more labile pheophytin, a methanol solution of chlorophyll *d* was cooled to  $-80^{\circ}$  and treated with hydrochloric acid dissolved in methanol. After 12 minutes the solution was neutralized with cold dimethylaniline which had been dissolved in a mixture of methanol and petroleum ether. The pigment mixture was warmed to room temperature and immediately transferred to petroleum ether. (When permitted to stand at room temperature, particularly in the presence of light, pheophytin solutions in methanol containing considerable quantities of dimethylaniline and water frequently developed

a deep purple-red color, due presumably to alteration of the dimethylaniline.) The petroleum ether solution of pheophytin was washed with water and then adsorbed on powdered sugar. After development of the chromatogram with petroleum ether containing 0.1 per cent *n*-propanol and 0.5 per cent dimethylaniline the principal band consisted of a yellow-brown pigment, *pheophytin d*, which was less adsorbed than the original chlorophyll *d*. Below this band of pheophytin *d* there were traces of a gray band which resembled pheophytin *a*. Identical results were obtained in another preparation in which all steps prior to adsorption were carried out in an atmosphere of hydrogen. The absorption spectrum of pheophytin *d* in methanol is shown in Fig. 9. Since the pigment was somewhat unstable, the absorption curve is probably not highly accurate.

Chlorophyll *d*, treated at room temperature with hydrochloric acid in methanol and then neutralized with dimethylaniline, was transformed principally into the gray pigment which had been produced in traces by the low temperature treatment. In this case, only traces of the brown pheophytin *d* were observed. We propose the name *isopheophytin d* for this gray pigment. The absorption spectrum of isopheophytin *d* in methanol is shown in Fig. 9. Isopheophytin *d*, like pheophytin *a*, forms a purple-gray solution in methanol and a deep blue solution in methanol containing hydrochloric acid.

Isochlorophyll *d* treated with acid, either at 20° or at -80°, was converted almost entirely into the gray isopheophytin *d*.

Pheophytin *d* in methanol, when reacidified at room temperature and subsequently neutralized with dimethylaniline, was transformed almost completely into the gray isopheophytin *d*. Isopheophytin *d* was not reconverted into pheophytin *d* when allowed to stand in acid solution at -80° for 90 minutes.

Adsorption of a dried preparation of isopheophytin *d* which had stood in the dark in an evacuated desiccator for 2 weeks showed that the pigment had been transformed in part to the brown pheophytin *d*. A faint gray band, presumably *isopheophytin d'*, was also observed just below the principal gray band of isopheophytin *d*.

Preparations of pheophytin *d* were much less stable than those of isopheophytin *d*. Pheophytin *d* was readily transformed, upon standing, into a mixture containing isopheophytin *d*. A pheophytin corresponding to chlorophyll *d'* was not observed, but limited material precluded an adequate search for the hypothetical pheophytin *d'*.

To permit a comparison of the properties of isopheophytin *d* with pheophytin *a*, chlorophyll *a* was converted at room temperature into pheophytin *a* by the procedure described above. The absorption spectrum of pheophytin *a* in methanol is shown in Fig. 10, together with that of

## CHLOROPHYLL D OF RED ALGAE

isopheophytin *d*. The spectra of pheophytin *a* and isopheophytin *d* are remarkably similar, bearing the same relation to each other as the spectra of chlorophyll *a* and isochlorophyll *d* (Fig. 7). The curves for pheophytin *a* and isopheophytin *d* were very closely reproducible for different preparations of the pigments. Adsorbabilities of pheophytin *a* and isopheophytin *d* were compared on a column of powdered sugar,  $1 \times 12$  cm. The pigments were washed through the column with petroleum ether containing 0.1 per cent *n*-propanol, but no separation occurred. Evidently their adsorbabilities are remarkably similar. Despite the similarity of their absorption spectra and adsorbabilities, these two pigments are readily

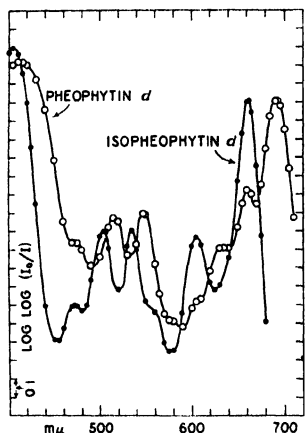


FIG. 9

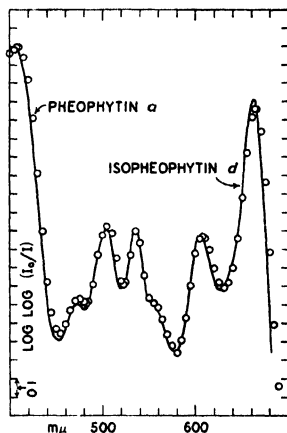


FIG. 10

FIG. 9. Absorption curve for isopheophytin *d* (dots) and approximate absorption curve for pheophytin *d* (circles). Solvent, methanol.

FIG. 10. Absorption curves for pheophytin *a* (circles) and isopheophytin *d* (line). Solvent, methanol.

distinguishable by their reactions with Grignard's reagent and with alkali and acid.

*Addition of Magnesium to Isopheophytin d and to Pheophytins d and a*—Methylmagnesium iodide dissolved in dry ether was added dropwise from a graduated pipette to a dry ether solution of isopheophytin *d*. Upon the appearance of a green color, an additional amount of the Grignard's reagent, equivalent to 3 times the original quantity, was added. After 4 minutes, an excess of cool 10 per cent sodium dihydrogen phosphate solution was added. The ether layer containing most of the pigment was separated and the aqueous layer was extracted again with additional ether. The combined ether extracts were washed with water and evapo-

rated nearly to dryness below room temperature at reduced pressure. Petroleum ether was then added and the resulting solution adsorbed on powdered sugar and washed with petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline. Six or more green or blue-green bands were visible on the column. The principal band was isochlorophyll *d*. The absorption spectrum for such a preparation is shown in Fig. 7. Little or no chlorophyll *d* or *d'* was formed directly from isopheophytin *d*, but both pigments were produced slowly by isomerization of the regenerated isochlorophyll *d*.

Pheophytin *d* treated in the same way with Grignard's reagent gave only very small yields of green or blue-green pigments. Little or no chlorophyll *d* or isochlorophyll *d* was formed. Considerable quantities of isopheophytin *d* were formed. A much more strongly adsorbed gray pigment was also formed ( $\lambda_{\text{max.}}$ , 659  $m\mu$  in methanol). This pigment, recovered and treated with Grignard's reagent, yielded a strongly adsorbed green pigment ( $\lambda_{\text{max.}}$ , 658  $m\mu$  in methanol) which was also found among the products formed by action of Grignard's reagent on isopheophytin *d* and on pheophytin *d*. Treatment of this strongly adsorbed green pigment with hydrochloric acid in methanol regenerated the strongly adsorbed gray pheophytin. The relation of this pheophytin to the other pheophytins described here is not clear.

Pheophytin *a* reacted with Grignard's reagent in a manner very similar to isopheophytin *d*, at least six or seven blue-green bands being observed on adsorption columns. Chlorophyll *a* was the principal product but constituted less than half of the total pigment mixture. Most of the products were adsorbed above chlorophyll *a*, which they resembled with respect to the wave-lengths of their absorption maxima. Formation of several chlorophyll *a*-like products, with a comparatively low yield of chlorophyll *a*, is in contrast with the report by earlier investigators of a yield of 0.8 gm. of chlorophyll *a* from 1.0 gm. of pheophytin *a* (13).

*Effect of Alkali on Chlorophyll and Pheophytin Isomers*—Fig. 6 gives the position of the principal absorption maximum in the red region of the spectrum for products formed by alkali treatment and subsequent acid treatment of chlorophyll *d*, isochlorophyll *d*, pheophytin *d*, and isopheophytin *d*. For comparison, data for chlorophyll *a* and pheophytin *a* are also included in Fig. 6. To avoid confusion, secondary maxima are not recorded.

For each pigment the following procedure was used. A solution of the chlorophyll or pheophytin in methanol was treated with 2 gm. of potassium hydroxide dissolved in methanol. The wave-length of the principal absorption maximum in the red region of the spectrum for the resulting solution was determined with a Bausch and Lomb visual spectrophotometer

(see Fig. 6). This solution was then treated with an excess of glacial acetic acid. Ether was added and the pigments transferred to the ether by addition of 10 per cent sodium chloride solution. The ether layer was separated, washed several times with water, and the position of the principal absorption maximum again observed. 1 or 2 ml. of concentrated HCl were then added to the ether and thoroughly mixed. This treatment removed most of the pigment from the ether layer. After about 5 minutes the pigment was retransferred to the ether by the addition of large quantities of sodium chloride solution. The ether solution was then washed thoroughly with water and the position of the principal absorption maximum again observed. It should be noted that in the last two cases the positions of the absorption maxima listed in Fig. 6 were determined in ether, while the determination for the first product of the alkali treatment was made in methanol solution containing potassium hydroxide.

From Fig. 6 it may be seen that, although the intermediate products were different in each case, the final product of this series of treatments appeared to be the same for chlorophyll *d* and its derivatives, so far as the position of the absorption maximum may be regarded as an indication of identity. None of these products was examined chromatographically. By contrast, the difference between the isochlorophyll *d* series and the chlorophyll *a* series was accentuated, rather than diminished, in the final products formed by alkali and acid.

#### DISCUSSION

In naming chlorophyll *d* and its derivatives, we have endeavored to use a system in harmony with the accepted nomenclature of the chlorophylls. The structure of chlorophyll *d* is obviously related to that of chlorophyll *a*. Extension of the term chlorophyll to such related green pigments appears logical and desirable.

In naming the isomers of chlorophyll *d*, it appeared desirable to retain the letter *d* as part of the designation. Numerals, either as subscripts or suffixes, were avoided, since they have been employed, in the case of chlorophylls *a* and *b*, to identify other types of products (14, 15). The use of the prime designation (chlorophyll *d'*), for an isomer with an absorption spectrum only slightly different from that of the parent substance, is in harmony with the designation for the corresponding isomers of chlorophylls *a* and *b* (11). For an isomer with an absorption spectrum greatly different from that of the parent substance, the prefix *iso* (isochlorophyll *d*) serves to give a distinguishing name while indicating the isomeric relation to the original pigment. Moreover, the prime designation combined with the prefix (isochlorophyll *d'*) then denotes a compound apparently related to isochlorophyll *d* in a manner corresponding to the relation of chlorophyll *d'* to chlorophyll *d*.

Extension of this system of nomenclature to the pheophytins of chlorophyll *d* and its isomers provides a convenient means of indicating analogous relationships between the isomeric pheophytins.

The striking similarities between isochlorophyll *d* and chlorophyll *a*, and between isopheophytin *d* and pheophytin *a*, both in physical properties and in chemical behavior, indicate that the molecular structures are very similar. So far as the chromophoric groups are concerned, the *a* and iso-*d* series of compounds must resemble each other much more closely than either resembles the *b* series. The adsorbabilities of chlorophyll *a* and isochlorophyll *d* are also very similar. Nevertheless, the difference in structure is sufficient so that the iso-*d* compounds are readily and reversibly converted to another series (the *d* series) which, at least superficially,

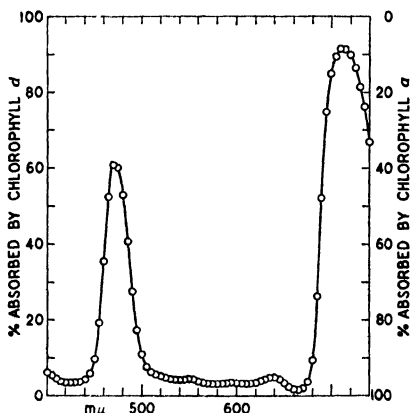


FIG 11 Relative proportions of light absorbed by chlorophylls *a* and *d* in a methanol extract of *Erythrophyllum delesserioides*. Absorption by red and yellow pigments is not considered in the calculation.

is very different from any product of chlorophyll *a* thus far observed. Furthermore, the *a* and iso-*d* series differ markedly in their reaction to successive treatment with alkali and acid (see Fig. 6).

The occurrence of chlorophyll *d* in red algae, together with the probable absence of chlorophylls *b* and *c*, is further evidence for the remoteness of any phylogenetic connection (9) between this large group of plants and most of the other groups of photosynthetic organisms.

The relative amounts of light absorbed by chlorophyll *d* and chlorophyll *a* in a methanol extract of *Erythrophyllum delesserioides* are shown in Fig. 11. If a similar relation prevails in the living organism, the large fraction of light absorbed by chlorophyll *d* at wave-lengths beyond 690 mμ may be of major significance to the plant. Neither phycoerythrin nor the carotenoids absorb appreciably at these wave-lengths. An investigation of



photosynthesis in *Erythrophyllum* in monochromatic red light should yield a clear cut answer regarding the photosynthetic effectiveness of chlorophyll *d*. If the light of long wave-lengths which is absorbed by this pigment can be utilized in photosynthesis, the presence of chlorophyll *d* must extend by at least 30  $m\mu$  the range of light utilized by plants in this fundamental process. The minimum energy per photon required to bring about the photochemical reaction (or reactions) in photosynthesis would accordingly be reduced by about 4 per cent.

Since water absorbs red light much more intensely than light of shorter wave-lengths, chlorophyll *d* in red algae can contribute little to absorption of red light except for plants growing near the surface. Brown algae (and other plants) are relatively transparent to light absorbed readily by chlorophyll *d*. Hence, for many red algae which grow on reefs in shallow water and which are shaded to a considerable extent by brown algae, light absorbed by chlorophyll *d* may amount to an appreciable fraction of the total energy available for photosynthesis.

#### SUMMARY

Various species of red algae contain, in addition to chlorophyll *a*, a second, green, magnesium-containing pigment, chlorophyll *d*. Neither chlorophyll *b* nor chlorophyll *c* was found in these algae.

Chlorophyll *d* was most easily prepared by adsorption of the pigments obtained by partial extraction of *Gigartina agardhii*. Maximum light absorption by chlorophyll *d* occurs at a wave-length longer than that of the maximum of chlorophyll *a*; in methanol, maximum absorption for chlorophyll *d* is at 696  $m\mu$ , compared with 665  $m\mu$  for chlorophyll *a*. Absorption at long wave-lengths by chlorophyll *d* may extend by 30  $m\mu$  the range of light utilized in photosynthesis.

Chlorophyll *d* was converted, rapidly upon heating or slowly at room temperature, into a mixture containing three isomers in addition to unaltered chlorophyll *d*. One of these isomers, chlorophyll *d'*, has an absorption spectrum very similar to that of chlorophyll *d*, whereas the other two isomers, isochlorophyll *d* and isochlorophyll *d'*, have spectra resembling the spectrum of chlorophyll *a*. The isomers were found to be reconvertible to chlorophyll *d*.

Treatment of chlorophyll *d* with acid removed the magnesium and formed a mixture of two interconvertible pheophytins. At  $-80^\circ$ , treatment with acid produced principally the labile yellow-brown pheophytin *d*. At room temperature, gray isopheophytin *d* was the principal product. Pheophytin *d* was rapidly converted to isopheophytin *d* when treated with acid at room temperature. Treatment of isochlorophyll *d* with acid, either at room temperature or at  $-80^\circ$ , produced isopheophytin *d*. Iso-

pheophytin *d* is remarkably similar to pheophytin *a* in its absorption spectrum and in its adsorbability on powdered sugar. Treatment of isopheophytin *d* with Grignard's reagent produced isochlorophyll *d* but little or no chlorophyll *d*. Neither chlorophyll *d* nor isochlorophyll *d* was formed by treatment of pheophytin *d* with Grignard's reagent.

When chlorophyll *d* and its isomers were treated successively with alkali and acid, the same final product was formed in each case. Chlorophyll *a* treated in the same manner yielded a product distinctly different from the chlorophyll *d* product.

We are indebted to the other members of the Division of Plant Biology for invaluable discussion and advice.

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# CRYSTALLINE MUSCLE PHOSPHORYLASE

## I PREPARATION, PROPERTIES, AND MOLECULAR WEIGHT\*

BY ARDA ALDEN GREEN AND GERTY T. CORI

(From the Department of Pharmacology, Washington University School of Medicine,  
St. Louis)

WITH A NOTE BY J. L. ONCLEY

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In 1936 glucose-1-phosphate was isolated from minced and washed frog muscle which had been incubated in phosphate buffer with traces of adenylic acid (1). It was shown that the ester was formed from glycogen by the reaction, glycogen + inorganic phosphate  $\rightarrow$  glucose-1-phosphate.

The enzyme which catalyzed this reaction was called phosphorylase, its presence in mammalian tissues (muscle, heart, brain, liver) and in yeast was demonstrated (2); phosphorylase was shown to play an important rôle in the formation of blood sugar in the liver (3).

In 1939 the reversibility of the reaction was shown for yeast phosphorylase by Kiessling (4) and for the mammalian phosphorylases by Cori, Schmidt, and Cori (5). In 1940 Hanes (6) described the presence of phosphorylase in peas and potatoes.

The polysaccharide synthesized by muscle phosphorylase resembles the amylose fraction of natural starch (7), while the enzymes from the other mammalian tissues (8) and from yeast (9) form a polysaccharide which resembles glycogen. Potato phosphorylase synthesizes a polysaccharide which resembles amylose (10). Cori and Cori (11) showed that phosphorylases of mammalian origin require the presence of polysaccharide for polysaccharide synthesis, and this was confirmed by Hanes (12) for plant phosphorylase. His claim that maltose could be substituted for polysaccharide was not confirmed by Green and Stumpf (13) nor by work done in this laboratory, when care was taken to free the maltose of polysaccharide impurities.

Cori, Colowick, and Cori (14) found that phosphorylases derived from vertebrate tissues required the addition of adenylic acid as coenzyme, while yeast (9) and potato (13) phosphorylases were shown to be active in the absence of added adenylic acid. Reducing agents (glutathione, cysteine, KCN) were shown to increase the activity of muscle phosphorylase (15), while they are without effect on potato phosphorylase (13).

The crystallization of muscle phosphorylase was announced in a pre-

\* This work was supported by a grant from the Rockefeller Foundation.

liminary note (16). The present paper deals with the method of preparation of the crystalline enzyme and some of its properties.

#### EXPERIMENTAL

*Preparation of Crystalline Phosphorylase*—Well fed rabbits are anesthetized by intravenous injection of pentobarbital and bled. Muscles of the back and legs are removed as rapidly and with as little stimulation as possible. All further steps are carried out in a cold room. The muscles are passed twice through a meat grinder, and extracted twice with an equal volume of ice-cold water. The water is stirred into the ground muscle and allowed to stand for about 10 minutes. The extract is strained off through gauze, filtered through cotton, and then through coarse filter paper.

The extract is adjusted to pH 6.0 to 6.2 with dilute HCl and dialyzed in cellophane tubes (Visking casings, diameter  $1\frac{1}{4}$  inches) against running cold water (5–10°) for 3 hours. It is then removed from the sacs and the pH brought to 5.8 to 5.9 with 0.03 N HCl. The pH is controlled with a glass electrode. An isoelectric precipitate forms which flocculates readily and settles rapidly on standing if the pH has been properly adjusted. The complete removal of this precipitate by centrifugation and filtration is an essential step in the method.

After neutralization of the perfectly clear supernatant fluid by the addition of about 1 gm. of sodium  $\beta$ -glycerophosphate per 100 cc. of solution, 0.7 volume of ammonium sulfate solution saturated at room temperature is added to make the final solution 41 per cent saturated (1.68 M). The ammonium sulfate should be neutral and the final pH 6.8. A relatively small quantity of precipitate forms which settles out overnight. After as much fluid as possible is decanted, this precipitate is collected by centrifugation. Centrifugation must be continued until the precipitate is well packed. 25 to 33 per cent of the protein in this precipitate is phosphorylase.

The ammonium sulfate precipitate obtained from the muscles of one rabbit is suspended in about 10 cc. of water and dialyzed against cold running water for 1 to 2 hours, during which time the proteins go into solution. Dialysis is continued against several changes of glycerophosphate-cysteine buffer of pH 6.8 (1 cc. of 0.3 M cysteine hydrochloride plus 39 cc. of 1 per cent sodium glycerophosphate). The cylinder containing the dialyzing bag and buffer solution is set in an ice bath. Crystals appear in a few hours or overnight, even when traces of ammonium sulfate are still present in the enzyme solution. If the phosphorylase content of this solution is very high, or if dialysis against water has been prolonged until protein begins to precipitate, this first precipitate may be amorphous, but reprecipitation in the following manner always yields crystals.

The crystals are centrifuged off in the cold and quickly dissolved at 30-35° in a solution of 0.03 M cysteine hydrochloride and 1 per cent sodium glycerophosphate (0.03 M) adjusted to pH 6.8. Insoluble material (which consists partly of cystine crystals) is centrifuged off and the clear solution transferred to an ice bath, whereupon phosphorylase crystals reappear, usually within 30 minutes. This alternate solution and crystallization by means of temperature changes may be repeated a number of times without undue loss of material.

In place of glycerophosphate, 0.03 M KCl may be used. Cysteine has the advantage of making the enzyme protein soluble in the presence of low

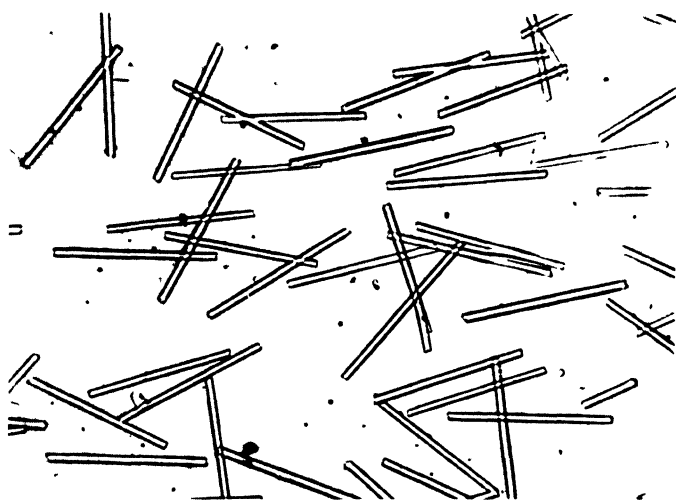


FIG. 1. Phosphorylase crystals prepared from rabbit muscle,  $\times 135$

concentrations of neutral salt, a condition which favors crystallization. Solutions containing 1 per cent or more of enzyme protein at 25° may be prepared in the presence of cysteine. The enzyme can be recrystallized from 0.5 to 1 M KCl solutions in the absence of cysteine, but its solubility even at this salt concentration is very low.

When crystallized from a concentrated solution, the crystals are small, sharp pointed needles which may appear as rosettes. This is the form which usually appears upon first crystallization. Upon slower crystallization from a more dilute solution, long blunt edged needles are formed, having the appearance shown in Fig. 1.

*Yield and Degree of Purification*—The effectiveness of the different steps of the preparation is illustrated in Table I. In the dialyzed crude extract

the phosphorylase protein constitutes about 2 per cent of the total protein.<sup>1</sup> The isoelectric precipitation removes about 11 per cent of the protein with relatively little loss of phosphorylase activity. In this step an enzyme is removed which would otherwise act on phosphorylase in later stages of the preparation and convert it into a form which does not crystallize under the above conditions.

TABLE I

*Sample Protocol of Preparation of Crystalline Rabbit Muscle Phosphorylase*

Preparation 69 The amount of phosphorylase protein present was calculated from activity measurements, as described in the text.

	Total protein	Phosphorylase protein	
	mg	mg	per cent
1 210 gm back muscle extracted twice with 210 cc H <sub>2</sub> O; filtered extract pH 6.5, 300 cc	4780		
2 Acidified with dilute HCl to pH 6.15 and dialyzed 3 hrs., 325 cc	4400	86	2
3 Acidified with dilute HCl to pH 5.85 and isoelectric ppt. removed, 313 cc	3910	71	2
4 Pptd. with 41% saturated (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> in 1% glycerophosphate buffer, pH 6.8, ppt. suspended in H <sub>2</sub> O and dialyzed, 8 cc	147	58	39
5 Dialyzed against glycerophosphate-cysteine buffer, pH 6.8, crystals centrifuged off and dissolved in buffer, 3.7 cc	58	52	90
6 Mother liquor, 6.9 cc	85	5	6
7 Muscle residue of (1) extracted with 210 cc 1% sodium glycerophosphate, extract pH 6.7, 200 cc	1310	13	1
8 Muscle residue of (7) extracted with 210 cc 1% sodium glycerophosphate, extract pH 7, 216 cc	696	13	2
9 Total yield of phosphorylase in 4 extractions		112	
10 Phosphorylase per 100 gm muscle		53	

The main step which leads to concentration and purification of the enzyme is the precipitation with 41 per cent saturated ammonium sulfate. In this step over 90 per cent of other proteins is discarded in the supernatant.

<sup>1</sup> The dialyzed crude extract can be diluted 50 times with 0.015 M cysteine for activity determinations. In this dilution phosphoglucosylase (17) does not interfere, because the Mg<sup>++</sup> ion concentration is too low to activate the enzyme. The calculation of the amount of phosphorylase protein present is based on the activity of the pure enzyme which corresponds to 3500 units per mg. The activity tests are described in Paper III of this series. Protein was determined by the method of Robinson and Hogden (18). A serum protein solution, analyzed by the micro-Kjeldahl method, was used as standard and was checked against a phosphorylase solution of known protein content. The phosphorylase protein contains 15.9 per cent nitrogen.

fluid, while the precipitate consists of about one-third pure phosphorylase. One crystallization raises the level of purity to about 90 per cent with only a small loss of enzyme in the mother liquor. In Preparation 69, Table I, 86 mg. of phosphorylase were present in the crude extract, of which 52 mg. or 60 per cent was recovered in the crystals.

The extraction of phosphorylase from muscle with 2 volumes of water is incomplete. A third and fourth extraction of the muscle residue with 1 per cent glycerophosphate yields additional enzyme, as shown in Table I. Under the assumption that the four extractions were exhaustive, the back muscles contained 53 mg. of phosphorylase per 100 gm. of muscle. The leg muscles of the same animal contained 75 mg. of phosphorylase per 100 gm. of muscle. The amount of enzyme present in the muscles of different

TABLE II  
*Effect of Successive Crystallizations*

Preparation 16. Upon each recrystallization the crystals were dissolved in a volume of buffer solution to give about the same concentration of protein per cc.

Crystallization No	Protein per cc		Activity per mg protein		Activity without added adenylic acid	
	Crystals	Mother liquor	Crystals	Mother liquor	Crystals	Mother liquor
	mg.	mg	units*	units*	per cent	per cent
0†	11 3		850		50	
1	4 6	9 6	3070	300	55	51
2	4 9	1 5	3140	1460	57	54
3	4 2	0 8	3180	2350	59	57
4	4 3	0 4	3570	2860	60	60

\* Units are in terms of a first order reaction constant, as described in Paper III.

† Dissolved and dialyzed ammonium sulfate precipitate.

rabbits varies by as much as 100 per cent. In four consecutive preparations the values were 81, 59, 40, and 62 mg. of phosphorylase per 100 gm. of muscle. The muscles of well fed rabbits seem to contain more enzyme than those of fasted rabbits.

The purification achieved by the first and by successive crystallizations is illustrated in Table II. The first crystallization increased the activity of the enzyme from 850 to 3070 units per mg. of protein, which corresponds to a change of purity level from 24 to 86 per cent. After three to four recrystallizations, the activity per mg. of protein remains constant within the limit of error of the method of testing.

The activity per mg. of protein of the mother liquor of the first crystals is very low. With each recrystallization, the purity level of the mother liquors rises until it approaches that of the crystals. The amount of



protein remaining in the mother liquors diminishes with each recrystallization until a stage is reached where the mother liquor contains dissolved enzyme protein only.

The last two columns of Table II show the activity of the enzyme without added adenylic acid, the activity in the presence of an optimal amount of adenylic acid being taken as 100. On an average, phosphorylase after several recrystallizations has 60 to 70 per cent of the activity obtained in the presence of adenylic acid. Phosphorylase can be obtained in another form which is inactive without addition of adenylic acid and which does not crystallize under the above conditions. This form predominates when

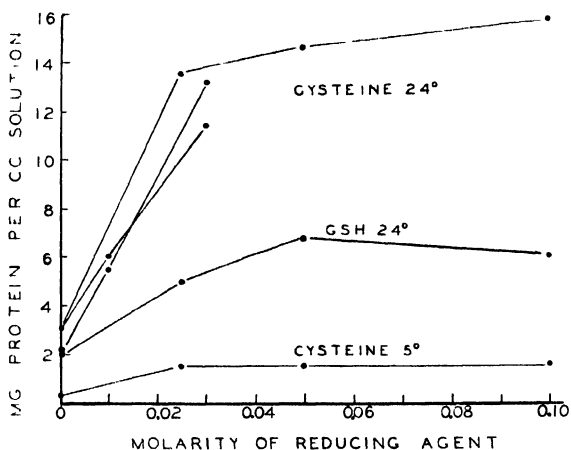


FIG. 2. Effect of cysteine and glutathione (GSH) on solubility of crystalline phosphorylase at pH 6.8. KCl was added, as necessary, to make all solutions 0.1 M with respect to total salt concentration.

the isoelectric precipitate at pH 5.8 (step (3) in Table I) is not removed. The significance of these findings will be discussed in Paper II of this series.

*Effect of Cysteine on Solubility*—Reducing agents (cysteine > glutathione > KCN) markedly increase the solubility of crystalline muscle phosphorylase. The high temperature coefficient of solubility in the presence of cysteine is made use of in the crystallization of the enzyme.

In Fig. 2 is shown an example of the effect of cysteine on solubility at two different temperatures and of glutathione at one temperature. In order to free the crystals of cysteine, they were washed repeatedly with ice-cold 0.03 M KCl in the centrifuge. To equal amounts of washed and drained crystals was added the same volume of solutions (pH 6.8) containing different concentrations of reducing agent and enough KCl to make the total salt concentration 0.1 M in all cases. The suspensions were

stirred frequently with a glass rod and kept for 4 hours at 24° or for 20 hours at 5°. After this time the undissolved protein was centrifuged off and the protein content determined in an aliquot of the supernatant fluid by the method of Robinson and Hogden (18). It seems probable that the effect of cysteine on solubility is related to the state of oxidation or reduction of the enzyme protein.

When the experiments were repeated with the same or with different enzyme preparations, the same type of curve was obtained in each case, but there was considerable variation in the total amount of protein dissolved at any given concentration of cysteine (see Fig. 2). These irregularities in solubility have not been explained and further studies on solubility seem indicated.

At constant cysteine concentration and pH the solubility of the enzyme is increased with increasing KCl concentrations in the manner characteristic of a globulin. The protein is so insoluble at low temperatures that so far it has not been possible to obtain solutions sufficiently concentrated for determination of the migration velocity in the Tiselius apparatus at the appropriate pH and ionic strength.

#### *Note on the Molecular Weight of Crystalline Phosphorylase*

By J. L. ONCLEY

*(From the Department of Physical Chemistry, Harvard Medical School, Boston)*

Solutions of about 0.5 and 0.25 per cent crystalline phosphorylase in a buffer composed of 1 per cent potassium chloride, 1 per cent sodium glycerophosphate, and 0.03 M cysteine hydrochloride neutralized to pH 7 have been used for measurements of sedimentation and diffusion constants. At 20° this buffer has a density of 1.0129 and a viscosity relative to water of 1.018.

The sedimentation constants were measured in an air-driven ultracentrifuge (19, 20) equipped with a modified Philpot schlieren optical system (21). The measurements were made in a cell 1.5 cm. high, 1.0 cm. thick, whose center was 6.5 cm. from the axis of rotation. A speed of 54,000 R.P.M., equivalent to centrifugal forces of from 200,000 to 240,000 times gravity, was used and the average temperature was about 24°. Values of the sedimentation constant have been reduced to the value in a solvent of the density and viscosity of water at 20°. Observed sedimentation constants,  $s_{20,w}$ , in 0.5 and 0.25 per cent solution were 13.3 and 13.6 Svedberg units, respectively, which would by linear extrapolation yield a value of about 13.7 at zero concentration. A few per cent of a much slower moving component ( $s_{20,w}$  about 4) was observed in the sedimentation diagrams of the more concentrated solution.

The diffusion constant was measured in a stainless steel Lamm type of diffusion cell (22), by means of the refractometric scale method (22). The temperature was maintained at 25°, and the reported diffusion constant values have been reduced to values in water at 20° ( $D_{20,w}$ ). Considerable difficulty in photographing the scale was encountered, owing to the formation of insoluble cystine. Only pictures taken after 625, 1192, and 2036 minutes could be used in evaluating the diffusion constant, these time intervals being somewhat less than would be used in an ideal experiment. The curves of refractive index gradient (line displacement) *versus* distance obtained at the two earlier times were quite symmetrical, but that obtained after 2036 minutes was rather skew, the displacements at a given distance from the original boundary on the solvent side being consistently greater. This skewness would not appear to be due to the variation of diffusion constant with concentration unless this is much larger than the variation in sedimentation constant. It may be due to complications arising from the settling of cystine from the solution.

Because of these complications, it is difficult to evaluate the diffusion constant with much accuracy. We have drawn the best possible symmetrical curve through the observed displacements, and calculated diffusion constants by the method of successive analysis ((22) equation 50), using displacement values ( $X$ ) of 60, 50, 40, 30, 20, and 10 per cent of  $X_{\max}$ . The average value for the diffusion constant,  $D_{20,w} \times 10^7$ , at 625 minutes was 3.2<sub>0</sub> with a mean variation of 2.0 per cent; at 1192 minutes, 3.3<sub>5</sub> with a mean variation of 2.4 per cent; and at 2036 minutes, 3.5<sub>4</sub> with a mean variation of 9.6 per cent. A value for the diffusion constant,  $D_{20,w} \times 10^7$ , between 3.2 and 3.8 would appear reasonable from these results.

In the absence of any measurement of partial specific volume, we have taken a value of 0.74 for tentative calculations of molecular weight and frictional ratio. When this value is combined with the above sedimentation and diffusion constants, we may estimate molecular weights of between 400,000 and 340,000, and frictional ratios,  $f/f_0$ , of between 1.4 and 1.2.

#### SUMMARY

1. The preparation of crystalline phosphorylase includes the following procedures: extraction of ground rabbit muscle with water, dialysis of the extract followed by removal of an isoelectric precipitate at pH 5.8, precipitation with 1.7 M ammonium sulfate at pH 6.8, and dialysis of the dissolved precipitate against a cysteine-glycerophosphate buffer at pH 6.8 and 0°. Crystals form after most of the ammonium sulfate has been dialyzed out. The crystals are separated and dissolved in cysteine-glycerophosphate buffer at 30°. Recrystallization is achieved by cooling the solution to 0°.

2. The enzyme protein constitutes about 2 per cent of the extracted proteins. About 60 per cent of the extracted phosphorylase is obtained in crystalline form.

3. Rabbit muscle contains 40 to 80 mg. of phosphorylase protein per 100 gm.

4. Cysteine greatly increases the solubility of the enzyme in weak salt solutions. Glutathione and KCN are less effective.

5. The molecular weight of crystalline rabbit muscle phosphorylase is between 340,000 and 400,000.

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# CRYSTALLINE MUSCLE PHOSPHORYLASE

## II. PROSTHETIC GROUP\*

By GERTY T. CORI AND ARDA ALDEN GREEN

*(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)*

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Phosphorylase has been obtained from muscle in two forms: as a euglobulin, phosphorylase *a*, which crystallizes readily and which has activity without addition of adenylic acid, and as a much more soluble protein, phosphorylase *b*, which is inactive without addition of adenylic acid. This second form has not yet been crystallized, but has been purified so that the best preparations show 90 per cent of the activity of form *a*. In Neuberg and Euler's nomenclature form *a* would be holophosphorylase, form *b* apophosphorylase.

It will be shown in this paper that muscle and other tissues contain an enzyme which converts form *a* to form *b* by splitting off the prosthetic group which apparently contains adenylic acid in combination with an as yet unidentified molecule. This enzyme will be referred to as PR (prosthetic group-removing) enzyme.

### EXPERIMENTAL

The presence of the PR enzyme in muscle was first suspected when it was found that a phosphorylase preparation obtained by direct ammonium sulfate precipitation of a water extract of muscle lost activity without added adenylic acid when it was kept at room temperature, while the activity with added adenylic acid remained unchanged. It was then found that the isoelectric precipitate at pH 5.8 to 5.9, which is described in Paper I, contains the PR enzyme. If the isoelectric precipitation is for some reason incomplete, PR enzyme is included in the ammonium sulfate precipitate and will act on phosphorylase during the subsequent dialysis, with the result that no crystals will be obtained. It is also clear that the manipulations preceding the isoelectric precipitation must be carried out as speedily as possible and that the temperature must be kept low throughout. Only when at least 30 per cent of the phosphorylase present in the ammonium sulfate precipitate is form *a* can crystals be obtained. The first crop of crystals may be contaminated with the PR enzyme and the preparation may be lost when recrystallization is attempted.

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Attempts to crystallize phosphorylase from heart muscle or liver by application of the procedure used for skeletal muscle have so far been unsuccessful, because the change from form *a* to *b* occurs so rapidly that it cannot be prevented by the methods now used.

Conditions are more favorable in skeletal muscle. In Preparations 68 and 69, Table I, the crude, dialyzed extracts contained only phosphorylase *a*. Table I shows that phosphorylase *a* is converted to some extent to

TABLE I

*Yield of Phosphorylase (Form a) from Resting Back Muscles of Rabbits*

The muscles were extracted with 2 volumes of water and the enzyme was isolated as described in Paper I. Measurements were made on the first crystals and the first mother liquor as well as on the dialyzed crude extract of the same muscles. Phosphorylase *a* has on an average 65 per cent of the activity obtained in the presence of optimal amounts of adenylic acid, the percentage of total phosphorylase which is form *a* was calculated on that basis. Phosphorylase *b* is inactive without addition of adenylic acid. For description, see the text.

Preparation No	Activity per mg protein with added adenylic acid		Activity without added adenylic acid		Phosphorylase <i>a</i> + <i>b</i> per 100 gm muscle		Phosphorylase <i>a</i>	
	Crystals	Mother liquor	Crystals	Mother liquor	Crystals	Mother liquor	Crystals	Mother liquor
	units*	units*	per cent	per cent	mg	mg	per cent	per cent
61	3070	290	51	27	36 1	2 9	79	42
63	3090	347	51	10	25 2	5.7	79	16
65	2140	139	65	60	14 7	1.3	100	93
68	2650	252	44	30	18.8	2.8	68	46
69	3150	193	52	42	24 8	2.2	80	66
68†	70		64		37.5		100	
69†	91		65		41 0		100	
67‡	115		10		48.0		16	

\* Units are in terms of a first order reaction constant, as described in Paper III.

† Dialyzed crude extract

‡ Previously stimulated muscle.

phosphorylase *b* in the course of the preparation of the crystals. The conclusion seems to be justified that resting muscle *in vivo* contains only phosphorylase *a*.

Preparations made from muscle which had been stimulated electrically or by strychnine before excision yield mainly phosphorylase *b*. Only one example (Preparation 67) is given in Table I, since the effect of stimulation will be discussed in greater detail in a later paper.

Previous work (1) carried out in this laboratory was mainly with phosphorylase *b*, since most of the preparations were inactive when tested without addition of adenylic acid. The method of preparation then used

did not involve the removal of the PR enzyme and it was undoubtedly the action of this enzyme and not the dialysis which rendered the preparations inactive without addition of adenylic acid.

Phosphorylase *a* is not converted to phosphorylase *b* (that is, the prosthetic group is not split off) by dilution, prolonged dialysis, washing of the crystals with cold dilute salt solutions, or exposure to the extremes of pH (pH 5.7 to 7.5) which the protein will tolerate without inactivation. Heating for 3 minutes to 52° at pH 6.8 causes almost complete inactivation of the enzyme, but does not lead to a splitting off of the prosthetic group in that part of the enzyme which survives this treatment. These observations indicate that the bond between the enzyme protein and the prosthetic group is not easily dissociated.

*Enzymatic Removal of Prosthetic Group*—The isoelectric precipitate obtained at pH 5.8 in the routine preparation of phosphorylase *a* was used as the source of the PR enzyme. The precipitate was dissolved with the aid of weak KOH and the turbid solution (pH about 7.5) was frozen. Upon thawing, a heavy precipitate could be removed, while the clear supernatant fluid retained PR activity. The PR enzyme was also prepared from spleen by the same method; per unit weight, spleen contained at least 10 times as much PR enzyme as muscle. The spleen enzyme could be purified by reprecipitation at pH 5.8.

In the test for the enzymatic removal of the prosthetic group a solution of phosphorylase *a* is incubated with PR or other enzymes at 25°. A control phosphorylase solution is incubated and tested under the same conditions. Aliquots are removed at different time intervals and phosphorylase activity with and without addition of adenylic acid is determined.

Phosphorylase *a*, when tested at pH 6.8, has on an average 65 per cent of the activity it has with added adenylic acid. This is illustrated in the fifth column of Table II, where the range for the control sample is 62 to 68 per cent. The last column shows that incubation with PR enzymes prepared from muscle or spleen leads to a progressive loss of activity in the absence of added adenylic acid, while when adenylic acid is added in the test, phosphorylase activity is not, or only slightly, below that of the control sample (fourth column).

The PR enzyme obviously does not destroy the phosphorylase protein during several hours of incubation, but changes it in such a manner that thereafter it requires the addition of adenylic acid for its activity. In the concentration at which it is available, the prosthetic group, once it is split off from the enzyme, obviously does not activate it. The assumption that phosphorylase *a* contains firmly bound adenylic acid and that the PR enzyme removes it from the protein would explain the experimental findings and has been adopted as a working hypothesis.



The PR preparation from spleen was found to possess considerable proteolytic activity at pH 6.8 in Anson's test (2) with hemoglobin as substrate, while a similar preparation of muscle which had less PR activity was also weaker in proteolytic activity. This suggested that the PR enzyme might split a peptide bond.

TABLE II

*Effect of Muscle and Spleen Protein Fraction (PR Enzyme) on Activity of Crystalline Phosphorylase*

PR preparations of different concentrations were added to phosphorylase dissolved in glycerophosphate-cysteine buffer at pH 6.8 and incubated at 25° for varying lengths of time before phosphorylase activity was tested in the presence and absence of adenylic acid.

Experiment No	Origin of PR enzyme	Time of incubation	Phosphorylase activity		
			With adenylic acid, incubated with PR	Without adenylic acid	
				Incubated without PR	Incubated with PR
		min	per cent*	per cent†	per cent†
1	Muscle	75	96	64	37
2	"	97	105	68	21
3	"	300	94	65	12
4	"	10		67	35
		33			10
		72			5
5	Spleen	60	98	65	22
		125	99	68	13
6	"	36	81	63	31
		100	91	64	8
7a	"	20	92	62	6
7b†	"	20	96	62	26
7b†	"	60	97	63	12

\* The activity of a control sample incubated without PR is taken as 100.

† The activity in the presence of adenylic acid is taken as 100.

‡ One-fourth the PR enzyme concentration of the sample in Experiment 7a

When crystalline trypsin or chymotrypsin was allowed to act on phosphorylase *a* at pH 6.8, the activity of the phosphorylase was progressively destroyed. It was noted, however, that the activity tested without addition of adenylic acid disappeared more rapidly than the activity with addition of adenylic acid. This indicated that the prosthetic group was split off more rapidly than the phosphorylase protein was destroyed and suggested the possibility of splitting off the prosthetic group without destroying the enzyme by incubating at a more acid pH.

In Table III it is shown that incubation of phosphorylase *a* with crystalline trypsin at pH 6.0 to 6.2 led to only a small loss in phosphorylase activity

when tested with adenylic acid (fourth column), while the activity without adenylic acid quickly dropped to low values (sixth column). Trypsin at pH 6 resembles the PR enzyme in that it removes the prosthetic group of phosphorylase while leaving the rest of the enzyme almost intact. It will be noted that in the control sample at pH 6 the percentage of activity without added adenylic acid is lower than at pH 6.8 (compare fifth column, Tables II and III).

TABLE III  
*Effect of Crystalline Trypsin and Chymotrypsin on Activity of Crystalline Phosphorylase*

Trypsin was added to 200  $\gamma$  of phosphorylase protein dissolved in glycerophosphate-cysteine buffer, pH 6 to 6.2, and incubated at 25°. After varying periods of incubation, phosphorylase activity was tested at pH 6.2 with and without added adenylic acid.

Experiment No	Trypsin added	Time of incubation	Phosphorylase activity		
			With adenylic acid, incubated with trypsin	Without adenylic acid	
				Incubated without trypsin	Incubated with trypsin
	$\gamma$	min	per cent*	per cent†	per cent†
1	1	10	90	44	28
2	5	10	109	56	27
	5	36	91	54	9
3	10	10	108	41	12
	10	26	90	41	9
	10	49	73	47	2
4	10	10	80	55	8
5	200	10	80	45	2
6	150‡	10	84	50	34
	150‡	32	63	58	27

\* The activity of a control sample incubated without trypsin is taken as 100

† The activity in the presence of adenylic acid is taken as 100.

‡ Chymotrypsin.

When phosphorylase *a* was incubated with crystalline chymotrypsin at pH 6 there was no marked difference in the loss of phosphorylase activity with and without addition of adenylic acid (Table III, Experiment 6). A cathepsin preparation of spleen (Anson) at pH 7 removed the prosthetic group and destroyed the phosphorylase protein at about the same rate.

Enzymes without effect on the activity of crystalline phosphorylase were carboxypeptidase, ribonuclease, and adenylic deaminase.

The nature of the bond between adenylic acid and the protein in phosphorylase *a* has not been elucidated. One would expect that the action of trypsin on phosphorylase *a* would consist in the splitting of a peptide bond.

An attempt was made to identify free adenylic acid in the digestion mixture of phosphorylase with trypsin. To 10 mg. of phosphorylase *a* was added 0.25 mg. of crystalline trypsin and the mixture incubated for 1 hour at pH 6. Before digestion with trypsin, the activity without added adenylic acid was 63 per cent of the activity in the presence of adenylic acid, while after digestion it was only 5 per cent. The mixture was adjusted to pH 7 and heated for 3 minutes at 100°. The clear supernatant was concentrated and tested for the presence of free adenylic acid by adding it to phosphorylase *a*. It was calculated that if 1 molecule of adenylic acid per 1 molecule of phosphorylase (molecular weight 400,000) had been freed by this treatment the concentrated material should have contained sufficient adenylic acid to result in a concentration of 10 micromoles per liter in the phosphorylase test. As shown in Fig. 5, Paper III, the test system is sensitive to 0.3 micromole of adenylic acid per liter and gives a nearly maximal effect with 10 micromoles per liter. Although the material gave a positive pentose reaction, there was no indication of the presence of free adenylic acid in the phosphorylase test. Negative results were also obtained when phosphorylase *a* was more completely digested by incubating it with both trypsin and chymotrypsin at pH 7 for 2 days.

Whatever is split off by trypsin cannot be free or unchanged adenylic acid. Molecular weight determinations have not yet been made on phosphorylase *b*. The molecular size of the prosthetic group also remains to be investigated.

It has not been possible to convert phosphorylase *b* to phosphorylase *a* by adding an excess of adenylic acid under varying conditions of pH, temperature, and presence and absence of reducing agents. Furthermore, the added adenylic acid could be dialyzed away completely. This indicates that the addition of adenylic acid to phosphorylase *b* does not result in the formation of an undissociable linkage such as is present in phosphorylase *a*. Nevertheless, the activity of phosphorylase *a* from which the prosthetic group has been completely removed by PR or by trypsin can be fully restored by addition of adenylic acid. It follows that a firm bond between adenylic acid and protein is not essential for the activity of the enzyme. The significance of the bound adenylic acid in phosphorylase *a* is probably twofold, (1) to make the enzyme self-contained, *i.e.* independent of the formation of adenylic acid by other reactions, and (2) to protect the adenylic acid from enzymatic destruction, for example by adenylic deaminase.

*Pentose and Phosphorus Content of Phosphorylase*—If adenylic acid is present in phosphorylase *a*, the enzyme should contain pentose and phosphorus. Pentose was determined by the colorimetric method of Mejsbaum (3) which is based on the Bial reaction and consists of heating with 1 per cent orcinol in concentrated HCl. In some cases 1 cc. of enzyme containing

4 to 6 mg. of protein was first treated with trichloroacetic acid, heated for 3 minutes at 100°, the protein precipitate removed by centrifugation, and the supernatant and precipitate were used for pentose determinations. Only a slight amount of the pentose color was given by the protein precipitate. In other cases 1 cc. of enzyme was analyzed without previous deproteinization. The strong HCl hydrolyzed most of the protein during development of the color (15 minutes at 100°). Pure adenylic acid was used as standard and the color was read in a Klett-Summerson photoelectric colorimeter with Filter 66.

Three phosphorylase *a* preparations which had been recrystallized four times gave values of 0.31, 0.30, and 0.26  $\gamma$  of pentose per mg. of protein, respectively. The last sample, when only twice recrystallized, gave a value of 0.6  $\gamma$  of pentose per mg. of protein. While no great accuracy can be claimed for these determinations, they are of interest in conjunction with the molecular weight determinations reported in Paper I. Assuming a 1:1 ratio, the average of 0.30  $\gamma$  of pentose per mg. of protein would correspond to a molecular weight of 500,000, a figure which is 25 per cent higher than that calculated from sedimentation and diffusion rates.

Highly purified phosphorylase *b*, when tested in the same amounts as phosphorylase *a*, did not give any measurable pentose reaction. The Molisch reaction was positive with phosphorylase *a* and negative with phosphorylase *b*.

For phosphorus determinations the crystals were first washed with large volumes of 0.03 M KCl in the cold and were then recrystallized several times from 0.03 M KCl plus 0.03 M cysteine. In other cases the enzyme after several recrystallizations was dialyzed for 2 days against frequent changes of distilled water. A sample of 3 to 4 mg. was ashed with sulfuric acid and hydrogen peroxide.

The average phosphorus content was 0.7  $\gamma$  per mg. of protein. This is much higher than would correspond to 1 molecule of adenylic acid per molecule of enzyme. There is the possibility that phosphorus is present in other combinations in the enzyme protein or that traces of impurities, for example phospholipids, still adhere to the enzyme. A highly purified preparation of phosphorylase *b* contained 0.2  $\gamma$  of phosphorus per mg. of protein. The presence of 1 molecule of adenylic acid per molecule of phosphorylase *a* could account only for a difference of about 0.1  $\gamma$  of P per mg. of protein.

#### SUMMARY

1. Phosphorylase has been obtained from muscle in two forms: as a crystalline euglobulin, form *a*, which has 60 to 70 per cent of its full activity without addition of adenylic acid, and as a more soluble amorphous pro-

tein, form *b*, which is inactive without added adenylic acid. Dilution, dialysis, or exposure to low or high pH or high temperature within the range in which the enzyme protein is not irreversibly denatured does not cause form *a* to lose its activity without added adenylic acid. In the presence of adenylic acid both forms of the enzyme have the same activity per mg. of protein.

2. Muscle contains an enzyme (PR) which can be separated from phosphorylase by isoelectric precipitation of a water extract at pH 5.9. If the PR enzyme is not removed, phosphorylase is obtained mainly as the amorphous form *b*.

3. Incubation of crystalline phosphorylase *a* with the PR enzyme of muscle or spleen converts it to form *b*; the phosphorylase activity in the presence of adenylic acid remains unchanged, while the activity without added adenylic acid disappears. The same result can be obtained by incubation with crystalline trypsin at pH 6. Carboxypeptidase, ribonuclease, and adenylic deaminase are without effect on crystalline phosphorylase.

4. It is concluded that crystalline phosphorylase contains adenylic acid in a prosthetic group which can be split off enzymatically. This is supported by pentose determinations: form *a* contains 0.3  $\gamma$  of pentose per mg. of protein, corresponding to a minimum molecular weight of 500,000, while form *b* does not give any measurable pentose or Molisch reaction. The material which is split off by the action of trypsin on crystalline phosphorylase gives a positive pentose reaction, but is not free adenylic acid.

5. Evidence is presented that resting muscle contains mainly form *a*; some form *b* arises during isolation of the enzyme, owing to unavoidable action of the PR enzyme. Previously stimulated muscle contains mainly form *b*, indicating an *in vivo* action of the PR enzyme.

The authors wish to thank Dr. Northrop and Dr. Kuntz for the supply of crystalline trypsin, chymotrypsin, carboxypeptidase, and ribonuclease, and Dr. Anson for a purified preparation of spleen cathepsin.

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# CRYSTALLINE MUSCLE PHOSPHORYLASE

## III. KINETICS\*

By CARL F. CORI, GERTY T. CORI, AND ARDA ALDEN GREEN

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

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Some observations of the kinetics of the reaction, glucose-1-phosphate  $\rightleftharpoons$  polysaccharide + inorganic phosphate, have been made with phosphorylase b (1). It seemed desirable to repeat and to extend these observations with crystalline phosphorylase a in order to see whether there is any difference between the action of the two forms of the enzyme.

### EXPERIMENTAL

Phosphorylase crystals are kept at 5° in 1 per cent glycerophosphate-0.03 M cysteine buffer of pH 6.7 in a concentration corresponding to 0.4 to 0.8 per cent protein. Under these conditions they retain their activity for several weeks. For activity tests an aliquot of the crystal suspension is diluted about 100 to 200 times with 0.03 M cysteine of pH 6.7. When this dilute solution is kept at room temperature and the activity is tested at various time intervals, there occurs a slight (5 to 15 per cent) rise in activity. This increase takes place within 5 to 20 minutes, and thereafter the activity remains constant for at least 1 hour. The activity of the enzyme is not increased further by doubling or tripling the cysteine concentration. The enzyme activity reaches the same peak in equimolar concentrations of cysteine, glutathione, or KCN, but the time required is longer for glutathione and still longer for KCN than for cysteine.

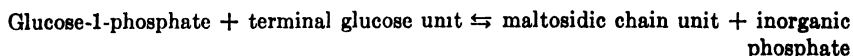
The usual composition of the reaction mixture was 0.016 M glucose-1-phosphate (1-ester), 1 per cent glycogen, and 0.015 M cysteine, pH 6.7, unless otherwise stated. The enzyme was added as the last component and the reaction mixture was incubated at 30°. Based on a molecular weight of 400,000, the enzyme concentration in the experiments reported in this paper was of the order of  $10^{-8}$  M. The crystalline enzyme has about 65 per cent of its maximal activity when no adenylic acid is added. Without addition of glycogen, the enzyme is completely inactive. The liberation of inorganic phosphate was used as a measure of the reaction to the right.

The pH optimum of the reaction is between 6.5 and 6.9. If the rate at pH 6.7 is taken as 100, it is 88 at pH 6.1 and 72 at pH 7.4. There occurs a shift in pH during incubation, because the 1-ester ( $pK_2$  6.1) which disappears is a stronger acid than the orthophosphate ( $pK_2$  6.8) which is formed.

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This change is small and has a negligible effect on the rate during the early course of the reaction. Glycerophosphate buffer (0.03 M) keeps the pH nearly constant, but it also decreases the activity of the enzyme. This will be illustrated later. A more suitable buffer, *i.e.* one which does not decrease enzyme activity, could not be found.

*Theoretical Considerations*—The polysaccharide formed by muscle phosphorylase is made up of a long, unbranched chain of glucose residues in 1:4 glucosidic linkage (2). The process of formation of this polysaccharide chain may be pictured as one of successive additions of glucose-1-phosphate units which exchange their ester bond for a glucosidic bond in the chain. The process would therefore be bimolecular; that is, 2 glucose molecules react, one representing the terminal unit of the chain and the other the glucose-1-phosphate unit to be added to the chain. As shown in the formulation given below, the reaction would also be bimolecular in the reverse direction.



A reaction from left to right does not occur when glucose-1-phosphate alone is added; that is, a polysaccharide chain cannot be started by a reaction between 2 glucose-1-phosphate molecules. The system needs another component. Either glycogen, which is a highly branched molecule, or the branched polysaccharide fraction of natural starch has to be present. The former contains about 9 per cent and the latter 4 per cent of terminal glucose units. Polysaccharide synthesis might consist in a lengthening of existing side chains by addition of glucose units in 1:4 glucosidic linkages.

The linear polysaccharides formed by muscle or potato phosphorylase *in vitro* and the linear fraction of natural starch (amylose) cannot be substituted for glycogen; they possess very few end-groups and are poorly soluble in water; they do not initiate polysaccharide synthesis by phosphorylase even in 0.1 per cent concentration. Owing to this fact, it is possible to determine the effect of glycogen concentration on the rate of the reaction to the right. With low concentrations (0.01 to 0.02 per cent) the system may fail to reach equilibrium; with intermediate concentrations the rate of the reaction falls off rapidly with time; with 0.5 to 1 per cent glycogen (and 0.016 M 1-ester) the reaction proceeds at nearly maximal rate and is kinetically of the first order. Such an effect of glycogen concentration would be expected if glycogen were one of the reacting molecules.

A special feature of the reaction as formulated is that the concentration of the terminal glucose units may be pictured as remaining constant as the length of the polysaccharide chain increases; hence the reaction could be kinetically of the first order. When the concentration of the terminal units

is much smaller than that of 1-ester, that is when only a small amount of glycogen is added, relatively few long chains would be expected to form, while the reverse would be true when a large amount of glycogen is added. The following calculation illustrates this. A 0.02 per cent glycogen solution contains about 0.002 per cent glucose residues as end-groups of side chains, which corresponds to a concentration of  $1.1 \times 10^{-4}$  M. If the original 1-ester concentration is  $1.4 \times 10^{-2}$  M,  $1.1 \times 10^{-2}$  M would be converted to polysaccharide when equilibrium is reached; hence the average chain length of the newly formed polysaccharide would be about 100 glucose units. With a higher glycogen concentration more side chains would be available as nuclei for polysaccharide synthesis and the average chain length would be shorter.

The interpretation of the kinetics of the reaction at low glycogen concentrations is based on the assumption that chain length is a limiting factor in the synthesis of the linear polysaccharide. As the chain length increases beyond a certain optimum, the rate of the reaction decreases more and more and may stop before equilibrium is reached. In the following the term "inhibition" is merely used as a simple way of describing this phenomenon.

*First Order Reaction and Definition of Units*—Under certain conditions which form the basis for the activity determination of the enzyme, the reaction is kinetically of the first order. The conditions necessary are constant pH, presence of a reducing agent, and 1 per cent of glycogen if the 1-ester concentration is 0.016 M. Phosphorylase *b* requires, in addition, the presence of adenylic acid. Under these conditions *K* is proportional to the enzyme concentration over a 10-fold range of enzyme dilutions.

$$K = \frac{1}{t} \log_{10} \frac{x_e}{x_e - x} \quad (1)$$

In Fig. 1 the experimental points fit closely a theoretical curve drawn according to Equation 1, not only for the initial part but also for the part of the curve which approaches equilibrium. The equation is that of a reversible first order reaction, where  $x_e$  is the per cent of 1-ester converted to polysaccharide at equilibrium and  $x$  is the per cent converted at a given time,  $t$  (in minutes). The value of  $x_e$  varies with pH; hence the pH of the reaction mixture must be known and kept constant. For a comparison of the catalytic activity of different enzyme preparations the initial 1-ester concentration and the temperature must be kept constant. For convenience, *K* multiplied by 1000 is termed "units" of enzyme present per 1 cc. of reaction mixture.

*Catalytic Constants*—1 mg. of phosphorylase *a* which has been recrystallized several times has an activity of 3500 units at pH 6.7 and 30° in a reaction mixture containing 0.016 M 1-ester, 1 per cent glycogen, 0.1 mM adenylic



acid, and 0.015 M cysteine. When 0.03 M glycerophosphate is used as buffer, the value is 3000 units per mg. In the former case, assuming a molecular weight of 400,000 for the enzyme, the initial rate of the reaction would correspond to a conversion of  $4 \times 10^4$  molecules of glucose-1-phosphate to polysaccharide per molecule of enzyme per minute.

*Second Order Reaction*—That the reaction can be kinetically of a higher order is shown when glucose, which is a competitive inhibitor, is added.

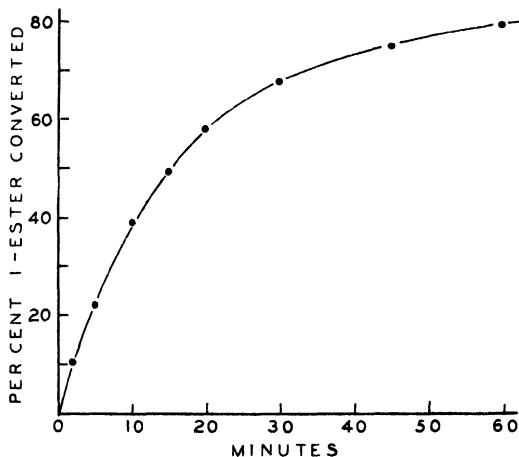


FIG. 1. Time curve for the conversion of 1-ester to polysaccharide. A glycerophosphate buffer of pH 6.7 was used. The curve, which has been drawn according to Equation 1, is of the first order.

In the presence of glucose the rate is that of a second order, reversible reaction and is described by Equation 2

$$K = \frac{x_e}{2ta(a - x_e)} \log_{10} \frac{x(a - 2x_e) + ax_e}{a(x_e - x)} \quad (2)$$

where  $a$  is the initial 1-ester concentration,  $x_e$  the amount changed at equilibrium, and  $x$  the amount changed at time,  $t$ . In testing the equation, the term to the left of  $\log_{10}$  was replaced by  $1/t$  in order to make the value of  $K$  independent of the units in which concentrations are expressed. An example is given in Table I. It may be seen that the position of the equilibrium is not changed by the addition of glucose and that the rate follows Equation 2 up to the point of approaching equilibrium.

In the presence of low concentrations of glycogen the reaction is slower than first order. Since the reaction approaches one of the first order with increasing glycogen concentration, one finds a family of transitional rate

curves, some of which are of the second order. An example of the rate of the reaction in the presence of 60 mg. per cent of glycogen is given in Table II; the agreement with Equation 2 is fairly satisfactory.

*Effect of Cysteine*—In order to free the enzyme of cysteine, phosphorylase crystals were washed several times with large volumes of 0.03 M KCl in the

TABLE I

*Polysaccharide Formation in Presence and Absence of Glucose*

The initial 1-ester concentration was 19.3 mm and the pH 7.1. The concentration of glycogen was 1 per cent.

Time	Without glucose		With 0.05 M glucose		
	1-Ester converted	$K \times 10^3$ , Equation 1	1-Ester converted	$K \times 10^3$ , Equation 1	$K \times 10^3$ , Equation 2
min	per cent		per cent		
5	44.2	75	32.7	49	26
10	63.1	76	50.7	47	28
15	71.1	76	59.6	44	28
26	75.6	74	68.5	38	27
40	76.5		73.1	33	26
60	76.5		75.0		

TABLE II

*Time Curve in Presence of Low Concentrations of Glycogen*

The initial 1-ester concentration was 12.6 mm and the pH 7.1. The glycogen concentration was 60 mg per 100 cc.

Time	1-Ester converted	$K \times 10^3$ , Equation 1	$K \times 10^3$ , Equation 2
min	per cent		
5	21.2	29	16
10	31.6	24	14
15	40.4	23	14
20	48.2	22	14
30	55.2	20	13
40	59.6	18	12
50	65.4	18	13
60	68.4	18	14
$\infty$	74.5		

cold. The crystals were dissolved in M KCl at room temperature, giving a solution of about 0.1 per cent protein. This enzyme preparation was tested without and with added cysteine. In the absence of cysteine the enzyme was only one-fourth to one-half as active as in the presence of 0.015 M cysteine. Fig. 2 shows that the rate curve was close to a straight line until equilibrium was approached. The position of the equilibrium was not

changed by the absence of cysteine. When cysteine was added to this enzyme preparation, the usual first order reaction rate was observed.

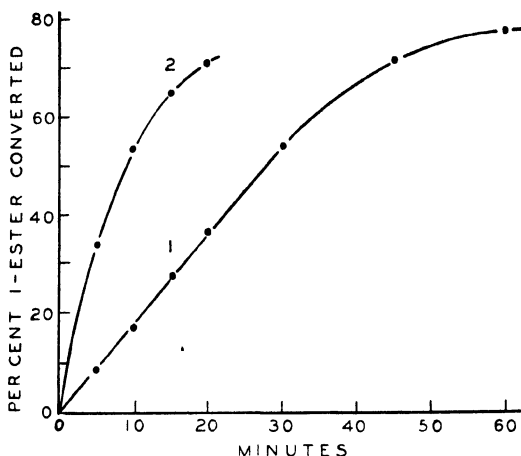


FIG. 2. Polysaccharide formation in the presence and absence of cysteine. Curve 1 without cysteine; Curve 2 with the same enzyme concentration and 0.015 M cysteine.

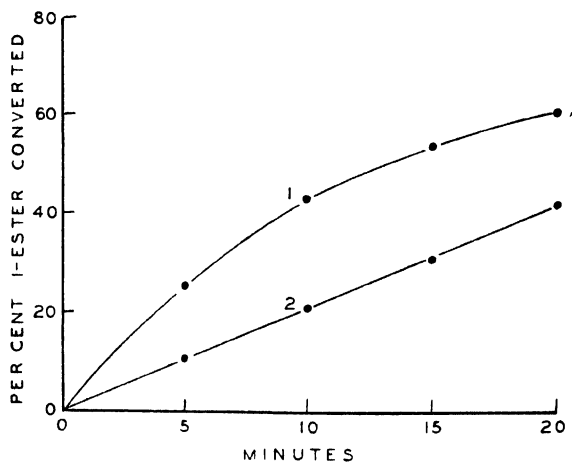


FIG. 3. Polysaccharide formation in the absence and presence of phlorhizin. Curve 1 without phlorhizin; Curve 2 0.0012 M phlorhizin.

*Effect of Phlorhizin*—When muscle phosphorylase is inhibited by phlorhizin, the early course of the reaction follows a straight line even in the presence of cysteine. An example is given in Fig. 3.

**Equilibrium**—The ratio, inorganic P to 1-ester P, at equilibrium varies with pH. This has been shown by Hanes (3) for potato phosphorylase and by Cori and Cori (1) for animal phosphorylases from brain, liver, and muscle. Although the above ratio decreases from about 7 at pH 6 to about 3 at pH 7, the ratios of the mono- and divalent ions of these two salts, as calculated recently by Hanes and Maskell (4), remain approximately constant over this pH range. The mean ratio of the monovalent ions is 10.6 for the plant and 10.2 for the animal enzymes, while the mean ratio of the divalent ions is 2.2 and 2.0, respectively. The equilibria of the two systems

TABLE III  
*Effect of pH on Position of Equilibrium*

For description, see the text.

pH	Inorganic P 1-Ester P	$x_e$ , Equation 1	Ratio, mono- valent ions	Ratio, divalent ions
7.33	2.45	71 0	9.7	1.98
7.30	2 39	70.5	9.4	1.92
6.96	3 11	75 7	10 0	2 04
6 94	3 22	76 3	10 3	2.11
6.53	3 81	79 2	8.8	1 80
6.51	4 08	80 3	9.3	1 90
6.07	5.90	85 5	9 3	1.89
6.07	6 81	87 2	10 9	2.22
6.14*	5.45	84 5	8 9	1 82
6.78*	3.59	78.2	10.2	2.09
7.25*	2.79	73.6	10.7	2 19
Average			9.8	2.0

\* After addition of adenylic acid.

appear to be very similar, although complete identity has not yet been established.

In the experiment in Table III small amounts of weak HCl or KOH were added in order to determine the position of the equilibrium at different pH in the same reaction mixture. The pH was determined with the glass electrode within 1 to 3 minutes of removing an aliquot for P analysis. Two analyses at different time intervals were made at each pH in order to insure that equilibrium had been reached. The values are given in terms of  $x_e$  of Equation 1, so that they may be used for the calculation of the first order velocity constant at different pH.  $x_e/(100 - x_e)$  equals the ratio inorganic P to 1-ester P. The ratio of the mono- and divalent ions was calculated from the dissociation constants of *o*-phosphoric acid ( $K_1$   $1.07 \times 10^{-2}$  and  $K_2$   $1.51 \times 10^{-7}$ ) and glucose-1-phosphoric acid (5) ( $K_1$   $7.8 \times$

$10^{-2}$  and  $K_2 7.4 \times 10^{-7}$ ). The mean ratio of the divalent ions at equilibrium was the same as that calculated by Hanes and Maskell from previously published data for animal phosphorylases.

*Temperature*—In order to reduce the time required for the attainment of temperature equilibrium to a minimum, the volume of the reaction mixture was 0.2 cc. The total time of exposure of the enzyme at each temperature was 8 minutes, 3 for the attainment of the temperature equilibrium and 5 for the reaction. The rates of reaction at  $14^\circ$ ,  $25.5^\circ$ ,  $39^\circ$ ,  $43^\circ$ , and  $49^\circ$  corresponded to 8.8, 48.0, 85.5, 52.9, and 7.8 units respectively. In another experiment at  $16^\circ$ ,  $25^\circ$ , and  $38.5^\circ$ , the rates were 12.8, 40.1, and 90.6 units. The reaction rate rises up to a temperature of  $39^\circ$  and falls off sharply at higher temperatures because of inactivation of the enzyme.

The energy of activation was calculated by means of the Arrhenius equation for the first temperature interval of each experiment and amounted to 25,300 and 24,300 calories per mole, respectively. When glucose-1-phosphate was hydrolyzed in 2.5 N sulfuric acid at  $25^\circ$  and  $35^\circ$ , values of  $2.0$  and  $9.1 \times 10^{-3}$  were obtained for the first order velocity constant, corresponding to an energy of activation of 27,500 calories per mole.

*Components of the System*—As shown previously (1), the combination of the enzyme with 1-ester, adenylic acid, and glycogen may all be expressed in terms of the Michaelis-Menten (6) equation or by the following equivalent expression

$$k = \frac{c(V - v)}{v} \quad (3)$$

where  $c$  is the concentration of the component added,  $V$  is the maximum rate at high concentrations of  $c$ , and  $v$  is the rate at a given concentration of  $c$ . The constant,  $k$ , corresponds to the value of  $c$  which gives half the maximal rate; i.e., when  $(V - v)/v = 1$ . Since Equation 3 is essentially a mass law expression,  $k$  has the dimensions of a dissociation constant.

Equation 3 may be rearranged in a linear form,

$$\frac{1}{v} = \frac{1}{V} + \frac{k}{V} \frac{1}{c} \quad (4)$$

The values for  $V$  are determined graphically by plotting  $1/v$  against  $1/c$  as proposed by Lineweaver and Burk (7). The velocity,  $v$ , is the initial velocity of the reaction and was evaluated by Michaelis and Menten as the tangent to the rate curve at its beginning. The proper choice of  $v$  as a measure of reaction velocity is therefore a matter of considerable importance and will be explained in each case.

*Glucose-1-phosphate*—When the 1-ester concentration is varied and all other components of the system are kept constant,  $v$  is expressed in terms of inorganic phosphate. The amount formed in 5 or 10 minutes is calculated

back to 0.5 minute by means of Equation 1 or 2, whichever applies, thus giving the initial rate of the reaction.

In Fig. 4 is given an example of the effect of three 1-ester concentrations in the presence and absence of glucose. It may be seen that in the absence of glucose a straight line results when  $1/v$  is plotted against  $1/c$ , as predicted by Equation 4. In the presence of glucose the same plot gives a curve concave to the  $x$ -axis, but when  $1/v$  is plotted against the reciprocal of the square of the 1-ester concentration, a straight line is obtained. This result was to be expected from the analysis of the experiment in Table I which showed that the rate curve is second order in the presence of glucose. The ordinate intercept,  $1/V$ , is the same in the presence and absence of glucose; this is characteristic of competitive inhibition.

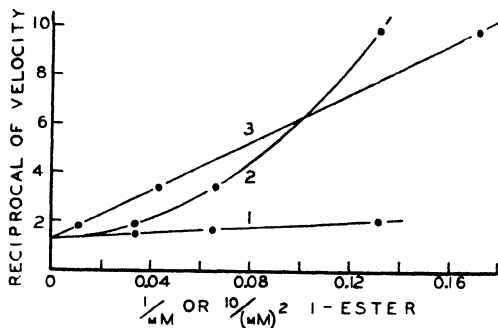


FIG. 4. Effect of 1-ester concentration in the absence and presence of glucose. Curve 1 without glucose, Curve 2 with 0.05 M glucose; both plotted against  $1/\text{mm}$  1-ester. Curve 3 with 0.05 M glucose, plotted against  $10/(\text{mm})^2$  1-ester. Glucose shows competitive inhibition; in its presence the reaction is second order; in its absence the reaction is of the first order.

Calculated from Equation 3 for Curve 1 in Fig. 4,  $k$  amounts to  $5.7 \times 10^{-3}$  M glucose-1-phosphate. There was some variation in the value of  $k$  (from  $4.8$  to  $6.4 \times 10^{-3}$  M) for different enzyme preparations. For a given enzyme preparation the same value of  $k$  was obtained whether or not adenylic acid was added. When 0.03 M glycerophosphate was used as buffer, the value of  $k$  was significantly lower, owing to a greater inhibition of the reaction by glycerophosphate at high than at low 1-ester concentrations.

**Adenylic Acid**—As stated in Paper II of this series, phosphorylase  $a$  is assumed to contain adenylic acid. Although firmly bound in a prosthetic group, this adenylic acid could nevertheless form a dissociable linkage with the enzyme protein through one of its phosphate groups. Such an internal dissociation is one possible explanation for the fact that addition of adenylic acid increases the activity of phosphorylase  $a$ .

When the prosthetic group is removed by the PR enzyme or by trypsin,

the catalytic activity can be fully restored by addition of adenylic acid. It was therefore of interest to compare the adenylic acid concentrations which have an effect on these two forms of enzyme.

A curve for the increase in rate with adenylic acid added to phosphorylase *a* is shown in Fig. 5. The rate has been calculated from a determination of the first order velocity constant and is expressed in per cent of *V*, the maximal rate in the presence of large amounts of adenylic acid. *V* has been obtained from a plot of  $1/v$  against  $1/c$ . Without added adenylic acid the enzyme showed 71 per cent of the maximal activity. The curve follows Equation 3 if the activity without added adenylic acid is taken as zero; *k* amounts to  $1.5 \times 10^{-6}$  M adenylic acid at pH 6.7 and 25°.

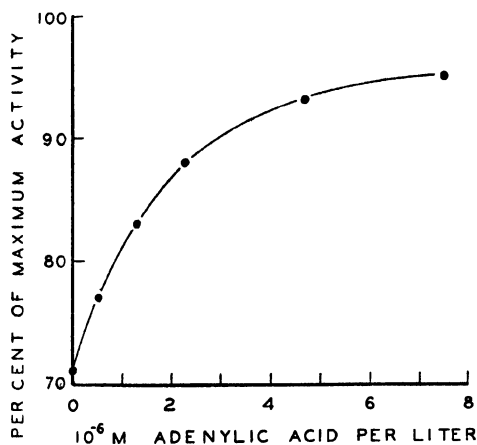


Fig. 5. Effect of adenylic acid on activity of crystalline phosphorylase

The dissociation constant for added adenylic acid is 30 times smaller with phosphorylase *a* than with phosphorylase *b*. The previously determined value for brain phosphorylase *b* was  $3 \times 10^{-5}$  M at pH 6.9 and 25° (1). A new determination on a muscle phosphorylase *b* preparation of 70 per cent purity gave  $5 \times 10^{-5}$  M at pH 6.7. It seems that the dissociation constant for phosphorylase *b* rises with decrease in pH, but this has not been fully investigated.

It has been reported previously (8) that adenosine diphosphate (but not adenosine triphosphate) can act as coenzyme of phosphorylase and that it is about one-half as active as adenylic acid. It has since been found that this result was due to the action of myokinase (9). This enzyme, which establishes the equilibrium,  $2 \text{ adenosine diphosphate} \rightleftharpoons \text{adenylic acid} + \text{adenosine triphosphate}$ , was originally detected in a purified phosphorylase preparation of muscle. Myokinase, which acts in extremely small amounts,

is removed by crystallization of phosphorylase. In the absence of myokinase, adenosine diphosphate does not activate phosphorylase. Inosinic acid has a weak activating effect when added in high concentrations. Phosphorylase *a* is therefore a highly specific and very sensitive test substance for the detection of adenylic acid.

*Glycogen*—In Table IV is given an example of the type of data needed for the evaluation of the constant for the glycogen-enzyme combination. In the presence of low concentrations of glycogen the first order velocity constant (expressed in units) falls off with time. As has been discussed above, this inhibition of the reaction may be the result of increasing chain length of the newly formed polysaccharide. In order to evaluate the constant for the glycogen combination, it is necessary to obtain the initial

TABLE IV  
*Effect of Glycogen Concentration*

Time	Glycogen added, mg per cent										
	1000		46		23		11.5		46	23	11.5
	1-Ester converted	Units	1-Ester converted	Units	1-Ester converted	Units	1-Ester converted	Units	<i>k</i> (Equation 3, $V = 73.3$ )		
	<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>		<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
0*		71.8		50.2		38.5		26.0	21.2	20.8	20.9
5	45.0	71.8	25.9	34.1	18.0	22.1	12.8	15.1	53	53	45
10	64.6	71.6	35.5	25.5	24.1	15.5	17.1	10.5	86	86	79
20			45.5	18.3	31.4	10.8	21.4	6.8	138	133	112

\* By interpolation from a graph of 1/units *versus* minutes.

rate. This can be done by graphic interpolation by plotting the reciprocal of the units against time. The ordinate intercept in such a graph represents the velocity of the reaction at zero time; that is, when no inhibition is present.

When the reciprocal of the rate at zero time is plotted against the reciprocal of the glycogen concentration, a straight line is obtained and the ordinate intercept is  $1/V$  of Equation 4. The constants may now be calculated as shown in Table IV. The constants for the zero minute rate hold over the whole range of glycogen concentrations, including the highest concentration of 1000 mg. per cent; *e.g.*,  $k = (73.3 - 71.8)/71.8 \times 1000 = 21$  mg. per cent. According to the present concept the terminal glucose residues of the glycogen molecule would be the units for combination with the enzyme. The value of  $k$  of 21 mg. per cent corresponds to a concentration of terminal glucose units of about  $1.2 \times 10^{-4}$  M. The enzyme concentration in this experiment was  $5 \times 10^{-5}$  M.



The progressive increase with time in the value of  $k$  which is shown in Table IV could be interpreted to mean that the more linear polysaccharide is formed the more glycogen is needed to saturate one-half of the enzyme. It should, therefore, be possible to overcome an existing inhibition by adding an additional amount of glycogen. Such an experiment with a phosphorylase *b* preparation of muscle has been recorded previously (1), but no detailed analysis was given.

The first part of the curve in Fig. 6 shows that the reaction in the presence of low concentrations of glycogen becomes so slow that equilibrium would not be reached in many hours. As soon as a sufficient amount of glycogen

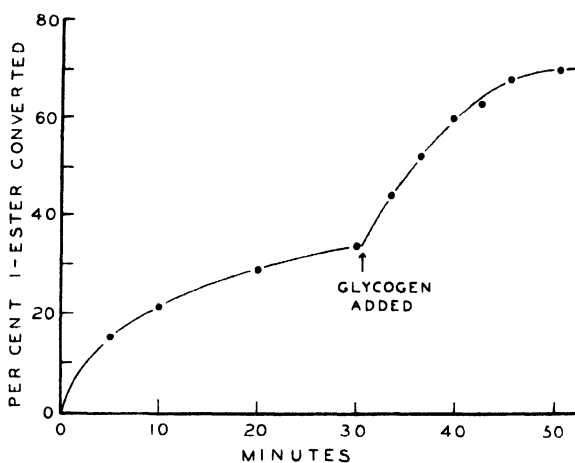


FIG. 6. Effect of glycogen on activity of phosphorylase. In the first part of the curve the glycogen concentration was 0.02 per cent. At the arrow the glycogen concentration was increased to 1 per cent.

is added, the rate becomes of the first order and equilibrium is approached rapidly. The first order constants were calculated by means of the equation,

$$K = \frac{1}{(t_2 - t_1)} \log_{10} \frac{(x_\infty - x_1)}{(x_\infty - x_2)} \quad (5)$$

where  $t_1$  is the time of addition of glycogen and  $x_1$  the amount of 1-ester which disappeared up to that time. The values for  $K \times 10^3$  were 38, 38, 39, 37, 39 for consecutive time periods. The same enzyme, when tested with 1 per cent glycogen added at the beginning, gave a first order constant of 37. This experiment shows that the enzyme regains its full catalytic activity when the inhibition is removed by addition of an optimal amount of glycogen.

The time required to reach equilibrium in the presence of low concentrations of glycogen depends on the enzyme concentration. This is illustrated in Table V. With the highest enzyme concentration it would have required about 3 hours for the reaction to reach equilibrium. Inspection of the data shows that each time the enzyme concentration was reduced one-half it took about twice as long to reach the same percentage of conversion of 1-ester. Hence, it would have taken 24 hours for the attainment of equilibrium with the lowest enzyme concentration. The experiment shows that in order to approach equilibrium in the presence of low concentrations of glycogen it is necessary to use very high enzyme concentrations. This is of importance if one wishes to prepare the linear polysaccharide with as little admixture of glycogen as possible. The "large scale" preparation of this polysaccharide has been described in another paper (2).

TABLE V

*Effect of Enzyme Concentration in Presence of Low Concentrations of Glycogen*

The glycogen concentration was 17 mg per 100 cc of reaction mixture and the pH 7.1.

Time	Enzyme dilution			
	1 200	1.100	1 50	1.25
	Per cent 1-ester converted			
<i>min</i>				
15	15 6	20.1	27.6	37 2
30	19 9	26.6	38 0	48 8
60	26 6	35.7	46 5	60 2
120	36 2	44 7	59 4	69 8

*Reversal of Reaction*—It is possible to reverse the reaction by addition of inorganic phosphate and to show that the linear polysaccharide can be converted back to 1-ester. For example, there were present per 1 cc. of reaction mixture 2.0 mg. of newly formed polysaccharide at the time of addition of inorganic phosphate. During subsequent incubation for 2 hours, inorganic phosphate disappeared and 1-ester was formed, corresponding to a disappearance of 0.76 mg. of polysaccharide. Since only 0.12 mg. of glycogen per cc. had been added, at least 0.64 mg. of polysaccharide was converted back to 1-ester. The linear polysaccharide fraction of natural starch can also be converted to glucose-1-phosphate. The slowness of both reactions is probably due to the low concentration of end-groups.

*Inhibitions*—The inhibitory substances investigated were glucose (10), phlorhizin, sodium  $\beta$ -glycerophosphate, and ammonium sulfate. In these experiments the inhibitor was added to the enzyme before the reaction was started by addition of 1-ester.

Glucose, as shown previously in this paper, causes competitive inhibition and in its presence the reaction is kinetically of the second order. With phlorhizin and the two salts the inhibition is of the non-competitive type.

A plot of the reciprocal of the rate against the glucose concentration gives a straight line, indicating that 1 molecule of glucose combines with the enzyme. In the case of phlorhizin a straight line results when the reciprocal of the velocity is plotted against the square of the phlorhizin concentration, indicating that 2 molecules of the inhibitor combine with the enzyme. This is illustrated in Fig. 7. In the case of the two salts, 1 molecule of inhibitor combines with the enzyme.

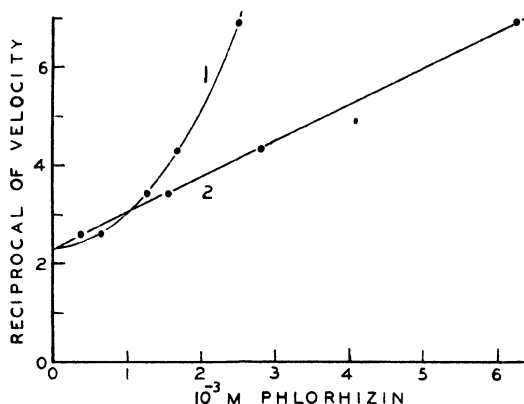


FIG. 7. Effect of phlorhizin concentration on phosphorylase activity. The reciprocal of the velocities is plotted against  $10^{-3}$  M phlorhizin in Curve 1 and against  $(10^{-3}$  M phlorhizin) $^2 \times 10^3$  in Curve 2. Curve 2 shows that 2 molecules of inhibitor combine with the enzyme.

*Effect of Adenylic Acid on Inhibitions*—The inhibition of crystalline phosphorylase by glucose and by phlorhizin is much greater in the absence than in the presence of adenylic acid. For example, with 0.02, 0.04, and 0.1 M glucose the inhibition was 15.6, 41.3, and 73.5 per cent respectively when no adenylic acid was added. In the presence of an optimal amount of adenylic acid 0.02 and 0.04 M glucose caused no inhibition, while 0.1 M glucose caused only 23.8 per cent inhibition. Similarly, with  $0.6$ ,  $1.2$ , and  $2.4 \times 10^{-3}$  M phlorhizin the inhibition was 14.1, 51.4, and 87.2 per cent respectively in the absence and 4.3, 23.2, and 60.4 per cent respectively in the presence of adenylic acid.

Very small amounts of adenylic acid are able to counteract the glucose inhibition. The experiment in Fig. 5 shows that addition of 2 micromoles of adenylic acid per liter has an intermediate effect and 8 micromoles a

nearly maximal effect on the activity of crystalline phosphorylase. The same concentrations of adenylic acid have a definite effect on glucose inhibition. For example, with 0.05 M glucose present in the reaction mixture, the per cent of 1-ester converted to polysaccharide was 23.2, 48.3, 51.4, and 51.6 when the concentrations of added adenylic acid were 0, 1.8, 7.5, and 30 micromoles per liter, respectively.

*Effect of Salts*—Data for sodium glycerophosphate and ammonium sulfate are presented in Fig. 8. The curves are rectangular hyperbolae and are

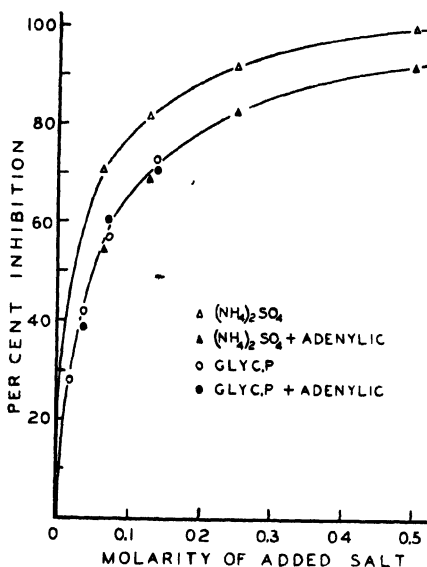


FIG. 8

FIG. 8. Inhibitory effect of ammonium sulfate and sodium  $\beta$ -glycerophosphate (glyc. P) on phosphorylase activity with and without addition of adenylic acid.

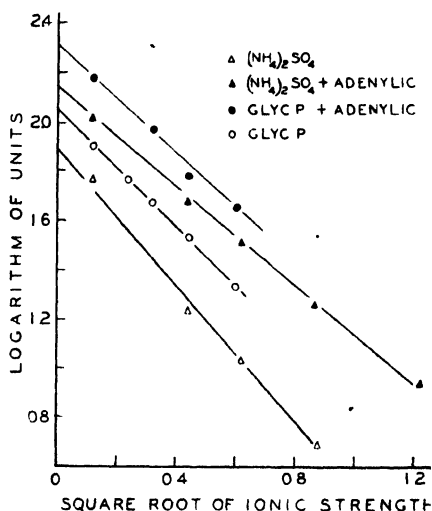


FIG. 9

FIG. 9. The data of Fig. 8 were plotted as logarithms of specific rate constant *versus* square root of ionic strength of added salt solution.

described by the equation,  $k = Iv/(v_0 - v)$ , where  $I$  is the concentration of the inhibiting salt,  $v$  is the rate in units for a given concentration of  $I$ , and  $v_0$  is the rate in the absence of inhibiting salt.  $k$  for glycerophosphate amounts to about 0.05 M per liter with or without added adenylic acid.

In the case of ammonium sulfate the values are 0.026 M in the absence and 0.051 M in the presence of adenylic acid. It seems clear that the presence of divalent neutral salts even in concentrations as low as 0.02 M can cause serious errors in activity determinations of the enzyme.

Fig. 9 shows how the effect of the neutral salts on the enzyme is related to

the ionic strength. A straight line with negative slope results when the logarithm of the first order velocity constant (expressed in units) is plotted against the square root of the ionic strength of the added salts.

#### SUMMARY

1. The reaction catalyzed by phosphorylase has been formulated as follows: glucose-1-phosphate + terminal glucose units  $\rightleftharpoons$  maltosidic chain units + inorganic phosphate. The terminal glucose units are the end-groups of the highly branched glycogen molecule. Without addition of glycogen no reaction to the right occurs; with low concentrations the reaction fails to reach equilibrium, with intermediate concentrations the rate of the reaction is kinetically of the second order; and with high concentrations of the first order. The theory is presented that glycogen enters into the reaction to the right and that polysaccharide synthesis consists in a lengthening of the side chains of glycogen by addition of glucose units in 1:4 glucosidic linkage.

2. Activity measurements are expressed in units and are based on a determination of the first order velocity constant under standard conditions. These are constant pH, presence of a reducing agent, and 1 per cent glycogen for a glucose-1-phosphate concentration of 0.016 M. 1 mg. of phosphorylase  $\alpha$  has an activity of 3500 units at pH 6.7 and 30°; this corresponds to a turnover number of  $4 \times 10^4$  glucose-1-phosphate molecules per molecule of enzyme per minute.

3. In the absence of a reducing agent the enzyme is only one-fourth to one-half as active as in the presence of 0.015 M cysteine and the early course of the reaction is zero order.

4. Although the ratio, inorganic P to 1-ester P, at equilibrium varies considerably with pH, the ratio of the divalent ions of these two acids remains constant at a value of about 2, confirming calculations of Hanes and Maskell. The pH optimum of the reaction is between 6.5 and 6.9.

5. The energy of activation amounts to 25,000 calories per mole between 15–25°. The rate of the reaction rises up to a temperature of 39° and falls off sharply at 43° because of inactivation of the enzyme.

6. The concentration at which one-half of the enzyme is combined with glucose-1-phosphate is  $5.5 \times 10^{-3}$  M. The corresponding value for glycogen is 20 mg. per 100 cc.

7. Addition of adenylic acid increases the activity of phosphorylase  $\alpha$ . When the activity without added adenylic acid is taken as zero, the dissociation constant of the added adenylic acid is  $1.5 \times 10^{-6}$  M at pH 6.7 and 25°. Form  $b$  of the enzyme, which is inactive without addition of adenylic acid, has a dissociation constant for the combination with adenylic acid of  $5 \times 10^{-5}$  M. Adenosine diphosphate does not activate phosphorylase if

myokinase is absent. Inosinic acid has a weak effect in high concentrations.

8. Glucose is a competitive inhibitor with respect to glucose-1-phosphate, while phlorhizin, ammonium sulfate, and sodium  $\beta$ -glycerophosphate are non-competitive inhibitors. In the case of phlorhizin 2 molecules combine with the enzyme, while in the case of the other inhibitors 1 molecule combines. The kinetics of the reaction are unchanged by the addition of ammonium sulfate and glycerophosphate. With phlorhizin the early course of the reaction is zero order; with glucose the rate of the reaction is of the second order. The inhibition by glucose, phlorhizin, and ammonium sulfate is counteracted by addition of small amounts of adenylic acid. A plot of the logarithm of the first order velocity constant against the square root of the ionic strength of added ammonium sulfate and glycerophosphate gives a straight line with a negative slope.

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# CRYSTALLINE MUSCLE PHOSPHORYLASE

## IV. FORMATION OF GLYCOGEN\*

By GERTY T. CORI AND CARL F. CORI

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

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It has been shown that glycogen is a highly branched molecule and that a 1:6 glucosidic linkage is present at the point of branching (1). Recent fractionation studies of natural starches have shown that two components can be separated (2-4). One (which is called amylopectin by Meyer (5)) has a branched structure and is similar to glycogen; the other, amylose, is made up of long, unbranched chains of glucose residues in 1:4 glucosidic linkage. Potato starch and corn-starch contain about 20 per cent amylose, the remainder being amylopectin.

The polysaccharides formed *in vitro* by brain, heart, and liver phosphorylase preparations give a brown color with iodine and resemble glycogen (6), while those formed by potato (7) and by muscle phosphorylase (8) have been shown to possess an unbranched structure and to resemble closely the amylose fraction of natural starch. If one assumes that muscle phosphorylase differs from the phosphorylases of other tissues, the fact that muscle contains glycogen and not amylose cannot be explained. Another possibility is to assume that all phosphorylases are of the type of the muscle enzyme; *i.e.*, that they can only form polysaccharides with 1:4 linkages and that another enzyme (or factor) is necessary to make the 1:6 linkages at the point of branching. The latter enzyme would be present in relatively high concentration in heart, liver, and brain extracts, while muscle extract (for an unknown reason) would contain very little of it.<sup>1</sup>

Attempts to free liver or heart phosphorylase of the second enzyme have not been successful. The best heart phosphorylase preparations had a purity level of only 20 per cent, if one assumes that pure muscle and heart

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<sup>1</sup> A recent observation is of interest in this connection. Waxy maize starch has been shown by Schoch (2) with the butanol fractionation method and by Bates *et al.* (3) with an iodine titration method to be free of the amylose component. The waxy maize starch gives a red-brown color with iodine. An extract was prepared from waxy maize pollen which was kindly supplied to us by Dr. Stadler. This extract formed a highly insoluble polysaccharide from glucose-1-phosphate which gave a pure blue color with iodine. The case is analogous to that of muscle which contains glycogen but yields an extract which forms amylose.



phosphorylases have the same activity per mg. of protein, and such preparations still formed a polysaccharide which gave a brown color with iodine. It has been possible, however, to carry out the reverse experiment; namely, to supplement muscle phosphorylase with a liver or heart extract which contained the second enzyme but which was devoid of phosphorylase activity. The combined action of these two enzymes resulted in the formation of a polysaccharide which resembled glycogen.

In contrast to glycogen and amylopectin, the polysaccharides formed by muscle or potato phosphorylase, as well as the amylose fraction of natural starch, do not "activate" phosphorylase. For this reason the kinetics of the reaction are not the same when a branched type of polysaccharide is being formed as when a linear type is being formed. The interpretation of some of the experiments reported in this paper is based on this difference in kinetics.

In studying the activity of crude preparations of brain, heart, and liver phosphorylase without added glycogen, there was noted an induction period followed by an S-shaped rate curve. Similar curves have been obtained with crude muscle phosphorylase preparations when used in high concentration and without addition of glycogen. This type of curve was ascribed to two factors: to the presence in the enzyme preparations of traces of glycogen which start the reaction, and to the catalytic effect of the newly formed polysaccharide. When somewhat purified, these phosphorylases remained inactive unless glycogen was added. In the case of brain, heart, and liver phosphorylase preparations it was sufficient to add 1 to 5 mg. of glycogen per 100 cc. of reaction mixture to start the reaction and to allow it to come to equilibrium (in the form of an S-shaped rate curve). In the case of purified or crystalline muscle phosphorylase the reaction does not reach equilibrium when such small amounts of glycogen are added because the polysaccharide formed by muscle phosphorylase alone does not have any activating effect on the enzyme. This has been discussed in some detail in Paper III of this series.

#### EXPERIMENTAL

Perfused livers of previously fasted rats and dog and rabbit hearts were extracted with 2 volumes of water. The extracts were dialyzed and an isoelectric precipitate which formed at pH 6 to 6.2 was removed. The supernatant fluid was precipitated with 40 per cent ammonium sulfate at pH 7 and the dissolved precipitate dialyzed against water and frozen. The precipitates formed on dialysis and on freezing were discarded and the clear supernatant fluid used for the experiments, in some cases after further fractionation with ammonium sulfate. The extracts showed no phosphorylase activity when tested without addition of adenylic acid.

When tested with the addition of adenylic acid, there was considerable phosphorylase activity, indicating the presence of phosphorylase *b*. These extracts were added to crystalline muscle phosphorylase and polysaccharide formation from 1-ester was tested in the absence of adenylic acid and presence of low initial glycogen concentration.<sup>2</sup>

An example with liver extract is given in Fig. 1. In this experiment 20 mg. per cent of glycogen were added to all samples. The liver extract alone did not form polysaccharide and the muscle phosphorylase alone showed the type of activity which is characteristic for low concentrations of glycogen. It may be seen that the simultaneous addition of phosphorylase and liver extract resulted in an S-shaped curve and that equilibrium was

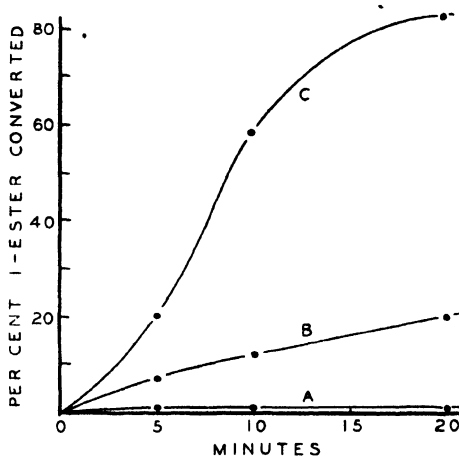


FIG. 1. Effect of liver extract on activity of muscle phosphorylase. All samples contained 0.016 M 1-ester, 0.015 M cysteine, and 20 mg. per cent of glycogen, pH 6.7. Curve A, liver extract alone, diluted 1:20; Curve B, phosphorylase alone, 56 units per cc.; Curve C, phosphorylase 56 units per cc. plus liver extract diluted 1:20.

reached in 20 minutes. The iodine color of the newly formed polysaccharide was reddish brown, whereas the iodine color when phosphorylase alone was used was pure blue. After this or other extracts were heated for 5 minutes to 100°, the activating effect on muscle phosphorylase was lost.

Still more striking effects were obtained when no activating glycogen was added (Fig. 2). In this case neither phosphorylase alone nor heart extract alone showed any measurable activity. When phosphorylase was

<sup>2</sup> The possibility that phosphorylase *b* might be activated by muscle phosphorylase *a* in the absence of adenylic acid was tested by letting the two enzymes act together in the presence of the optimal glycogen concentration. No activation of phosphorylase *b* took place.

combined with the heart extract, there was a pronounced induction period followed by an autocatalytic curve.

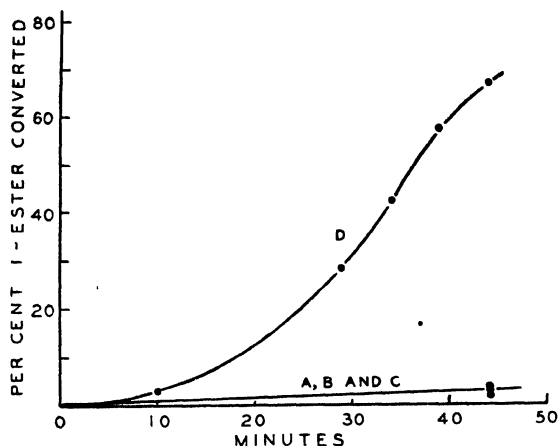


FIG. 2. Effect of heart extract on activity of muscle phosphorylase. All samples contained 0.016 M 1-ester and 0.015 M cysteine, pH 7; no glycogen was added. Curve A, heart extract alone, diluted 1:20; Curve B, phosphorylase alone, 40 units per cc.; Curve C, phosphorylase plus heated heart extract. Curve D, phosphorylase 40 units per cc. plus unheated heart extract diluted 1:20.

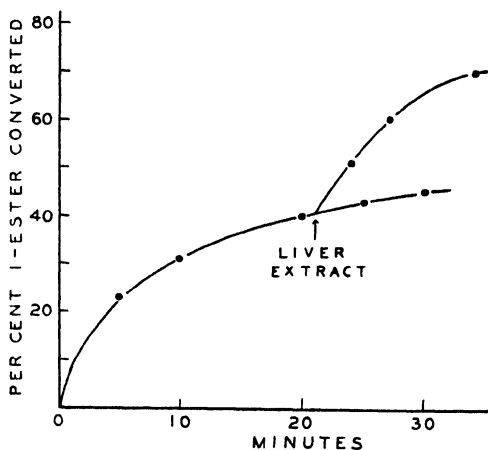


FIG. 3. Effect of liver extract at later stages of phosphorylase activity. The sample contained 0.016 M 1-ester, 0.015 M cysteine, and 20 mg. per cent of glycogen, pH 7. At the arrow, liver extract, final dilution 1:20, was added to an aliquot of the reaction mixture.

The experiment in Fig. 1 was modified by adding the liver extract after phosphorylase had been allowed to act for some time, so that the rate of the

reaction had become very slow (Fig. 3). Under these conditions the effect of addition of liver extract was very similar to that of addition of extra glycogen in the experiment in Fig. 6 of Paper III.

Since the tissue preparations contained diastase, it was of interest to see whether serum diastase would reproduce the effect of tissue extracts on phosphorylase. Addition of blood serum, in the absence of glycogen, did not start phosphorylase activity, in contrast to the effect of tissue extracts. In the presence of added glycogen, there was a slight activating effect, but the concentration of blood serum was rather critical (Table I). With 10 mg. per cent of glycogen and a 1:100 dilution of rat serum, there was an inhibition of phosphorylase activity; with a 1:200 dilution there was a transient stimulation; and with a 1:400 dilution there was a slight but persistent increase in activity. With higher concentrations of glycogen, the stimulatory effect was somewhat greater, provided the optimal concentra-

TABLE I  
*Effect of Rat Serum on Activity of Muscle Phosphorylase*

All samples contained 0.016 M 1-ester, 0.015 M cysteine, and 10 mg. per cent of glycogen.

Time  mins	Per cent 1-ester converted			
	Without serum	Dilution of added serum		
		1:100	1:200	1:400
5	21.6	16.4	20.2	20.5
10	24.3	16.6	30.3	27.0
20	32.6	17.0	29.7	38.8

tion of blood serum was chosen. An example is given in Fig. 4 which represents the largest effect with blood serum observed so far. It may be seen that Fig. 4 differs markedly from Fig. 1, both with respect to the magnitude of the effect and the type of curve which is obtained. Effects similar to those shown in Fig. 4 were also obtained with greatly diluted solutions of salivary diastase.

The effects of diastase may be pictured as follows: If too much serum is added, the diastase breaks down the activating glycogen and phosphorylase activity is inhibited. Within a narrow range of concentrations, however, diastase actually restores some of the originally present "activating" end-groups of the added glycogen by splitting off the newly formed long chains of glucose residues. An autocatalytic type of curve is not produced, because the addition of diastase does not lead to the formation of additional polysaccharide which activates phosphorylase. This is supported by the fact that no activating polysaccharide could be obtained by letting serum diastase act on the amylose fraction of natural starch.

The experiments reported in this paper show that when muscle phosphorylase is combined with a supplementary enzyme which is present in liver and heart extracts, an activating type of polysaccharide is formed. This polysaccharide was isolated in order to compare its activating power with that of glycogen.

A mixture containing 1-ester (22 mg. as glucose), muscle phosphorylase, supplementary enzyme from rabbit heart, and 0.2 mg. of glycogen in 8 cc. was incubated at 25° for 1 hour; 53 per cent of the 1-ester was split corresponding to the formation of 11.6 mg. of polysaccharide. The mixture was frozen and then centrifuged at 5° until the ice had melted. The pre-

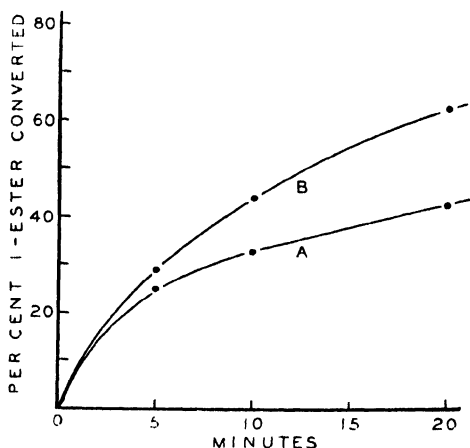


FIG 4 Effect of addition of blood serum on activity of muscle phosphorylase. All samples contained 0.016 M 1-ester, 0.015 M cysteine, and 20 mg. per cent of glycogen, pH 6.8. Curve A, phosphorylase alone; Curve B, phosphorylase plus rat serum, diluted 1:200.

cipitate, containing polysaccharide which gave a blue color with iodine, was discarded and the supernatant fluid precipitated with alcohol; after the precipitate was taken up with water, insoluble material (protein) was discarded. The alcohol precipitation was repeated twice; 5.8 mg. of polysaccharide were recovered. In a concentration of 30 mg. per 100 cc. of reaction mixture this polysaccharide had a somewhat greater activating power for muscle phosphorylase than an equal concentration of liver glycogen. In another experiment (in which the ratio of muscle phosphorylase to supplementary enzyme was varied) the activating power was less than that of glycogen. Further work seems necessary before the identity of this activating polysaccharide with glycogen can be established.

The question of the sequence of action of phosphorylase and the supplementary enzyme was investigated as follows: Amylose formed by muscle or potato phosphorylase *in vitro* and amylose isolated from natural starch were incubated with heart extract containing the supplementary enzyme. After varying periods of incubation, aliquots of the mixture were heated to destroy the enzyme and tested for the presence of activating polysaccharide by adding them to muscle phosphorylase; no activating polysaccharide could be detected. This suggests that phosphorylase and the supplementary enzyme must act simultaneously in order to produce an activating polysaccharide.

The nature of the supplementary enzyme has not been established. It might be another type of phosphorylase which can make 1:6 glucosidic linkages or it might be an enzyme related to the diastases. It seems fairly certain, however, that the diastase present in blood serum does not reproduce the effect obtained with liver or heart extracts.

#### SUMMARY

1. Muscle phosphorylase when acting alone converts glucose-1-phosphate to a polysaccharide which closely resembles the amylose fraction of natural starch, and consists of an unbranched chain of glucose residues in 1:4 linkage.
2. The simultaneous action of muscle phosphorylase and a supplementary enzyme obtained from heart or liver results in the formation of a polysaccharide which resembles glycogen.
3. The theory is presented that the formation of a branched type of polysaccharide (glycogen in animal tissues, amylopectin in plant tissues) is the result of the action of two enzymes.

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# PHOSPHATE EXCHANGE IN RESTING CARDIAC MUSCLE AS INDICATED BY RADIOACTIVITY STUDIES. IV\*

By ROBERT F. FURCHGOTT AND EPHRAIM SHORR

With the Technical Assistance of GLADYS BREWER

*(From the New York Hospital and the Department of Medicine, Cornell University Medical College, New York City)*

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In a preliminary group of experiments, which have been previously reported in brief (3), a study was made of the uptake of radioactive phosphorus by inorganic and organic phosphate compounds of cardiac muscle slices metabolizing at different levels of r.q. A rapid uptake of radioactivity by the various phosphate compounds of the tissue was observed and found to be of the same magnitude whether carbohydrate or fat was being oxidized as judged from the r.q. However, the data obtained were not susceptible to a strict quantitative interpretation because of the lack of certain fundamental information, in particular, with respect to the concentration and radioactivity of the intra- and extracellular fractions of the tissue inorganic phosphate. Although it was felt that the intracellular inorganic phosphate was the direct donator of radioactive phosphorus to the organic phosphate compounds, the available data only permitted a comparison of their uptake of radioactivity with that of the total tissue inorganic phosphate.

This necessary information was provided in the present experiments by a washing procedure which made possible separate analyses of the concentration and radioactivity of the extra- and intracellular fractions of the tissue inorganic phosphate. This procedure, along with others which were developed, revealed a marked difference in the uptake of radioactivity by these two portions of the tissue inorganic phosphate. They also provided evidence that the intracellular portion is the direct donator of phosphate to the organic phosphate compounds of the tissue, and that the quantitative turnover of phosphate through adenosine triphosphate and creatine phosphate in surviving cardiac tissue of the dog can be explained in accordance with modern concepts of the relation between phosphorylation and oxidation.

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### Methods

*Treatment of Tissue*—The cardiac tissue was obtained from normal dogs under nembutal anesthesia. Slices from the left ventricle about 0.5 mm. thick were aerated at room temperature in Ringer's solution with oxygen for 20 or 30 minutes, changes of the Ringer's solution being made every 10 minutes. The slices were then transferred to an Erlenmeyer flask for equilibration in a Ringer-M/75  $\text{PO}_4$  medium of pH 7.4. About 25 cc. of medium were used for every 4 gm. of tissue in a flask of 10 times the volume of the medium. The tissue was equilibrated in the Ringer- $\text{PO}_4$  medium under an atmosphere of oxygen at  $37.5^\circ$  by shaking in a Warburg bath for  $\frac{1}{2}$  hour (unless otherwise noted).

After the period of equilibration, radioactive P ( $\text{P}^*$ ) was added to the medium in the form of M/75 sodium phosphate (pH 7.4) in isotonic NaCl solution. The tissue was then allowed to incubate in this medium under the same conditions as in the equilibration for the desired length of time. Following incubation, tissue and medium were chilled to  $2^\circ$  and the rest of the procedure through the freezing of the tissue was carried out at that temperature in a cold room. In some experiments incubation was also carried out at  $2^\circ$  in a cold room, the tissue and medium being chilled in an ice bath immediately after the equilibration and before the addition of  $\text{P}^*\text{O}_4$ .

The first sample of tissue for analysis was taken after incubation and chilling. This was rapidly transferred to a small wire sieve and freed of adhering medium with three rinses of cold Ringer's solution. It was then immediately dried between two layers of towel, weighed, and dropped into a freezing mixture of dry ice and ether. The wet weight of each sample taken was generally about 4 gm.

The remaining tissue slices were then subjected to a thorough washing with phosphate-free Ringer's solution to remove the easily diffusible (extra-cellular) phosphate. The slices were continuously shaken throughout the washing, the Ringer's solution being replaced at intervals by 300 cc. of fresh solution. Samples of tissue were removed at desired intervals, and without further rinsing, were dried, weighed, and frozen.

For the extraction of the acid-soluble phosphates from the samples, each sample was first pulverized in the frozen state by means of an apparatus described by Graeser *et al.* (4). The pulverized frozen sample was mixed with ice-cold 5 per cent trichloroacetic acid in a 100 cc., stoppered, graduated cylinder to a total volume of 51 cc. (amounting to 50 cc. of fluid, on the basis of about 1 gm. of solids for every 4 gm. of tissue). This extraction mixture was shaken vigorously at intervals of several minutes over a period of  $\frac{1}{2}$  hour. It was then filtered in the ice box through a Whatman No. 42 filter.

*Chemical Procedures*—The trichloroacetic acid extracts were generally

analyzed for inorganic phosphate, the phosphate of creatine phosphate, and the labile phosphate of adenylyl pyrophosphate. In some experiments, the remainder of the organic acid-soluble phosphate, called here "residual phosphate," was also determined.

Two 10 cc. aliquots of the freshly filtered, cold trichloroacetic acid filtrate were added to standard 15 cc. conical centrifuge tubes, which were used throughout this study for the precipitation of the phosphate fractions. To each aliquot was added 0.6 cc. of concentrated  $\text{NH}_4\text{OH}$  and 1.0 cc. of a 15 per cent solution of  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ . The precipitation of the inorganic phosphate (IP) of the extract as  $\text{MgNH}_4\text{PO}_4$  was initiated by vigorous stirring with a glass rod. Precipitation was generally allowed to continue overnight in the ice box, although a few hours of standing appear to suffice for its completion. The  $\text{MgNH}_4\text{PO}_4$  was then separated from the supernatant fluid by centrifugation, washed twice with 3 cc. portions of dilute  $\text{NH}_4\text{OH}$  (1:10), and allowed to drain. It was then dissolved in 1 cc. of 0.1 N  $\text{HCl}$ , and made up to 10 cc. with water.

To 10 cc. of the supernatant fluid from the  $\text{MgNH}_4\text{PO}_4$  precipitate there were added, at room temperature, 0.3 cc. of concentrated  $\text{HNO}_3$  and 3.4 cc. of Embden's strychnine molybdate reagent (5), the components of which had been mixed at least 1 day previously. This acidified reagent split off the phosphate from the creatine phosphate (CP) and brought about its immediate precipitation as strychnine phosphomolybdate. Centrifugation was generally carried out 15 minutes after addition of the reagent, although precipitation is complete within 10 minutes. After centrifugation, the precipitate was washed once with 4 cc. of water, allowed to drain, dissolved in the minimal amount of 1 per cent  $\text{NaOH}$ , and brought to 10 cc. with water.

During the splitting of CP at room temperature, only an insignificant amount of adenylyl pyrophosphate (APP) is simultaneously hydrolyzed. To split the labile phosphate from the APP, a tube containing 10 cc. of the supernatant fluid from the precipitation of the phosphate of CP was placed in a boiling water bath for 5 minutes and then cooled in cold water. The split labile phosphate from the APP was precipitated during this procedure, probably largely as ammonium, rather than strychnine, phosphomolybdate because of the degradation of strychnine during the heating. The precipitate was then treated in the same way as the precipitate of the CP phosphate.

In experiments in which residual phosphate was also determined, 5 cc. of the supernatant fluid from the precipitation of phosphate of APP were ashed with 0.4 cc. of concentrated sulfuric acid in a 30 cc. Pyrex test-tube over a micro burner. As in the Cori procedure (6),  $\text{H}_2\text{O}_2$  was used to complete oxidation and a final boiling with added water (2 cc.) was employed to

split any pyrophosphate or metaphosphate formed. After cooling, the solution was transferred with two 2 cc. washings to a 15 cc. conical centrifuge tube. There it was neutralized with NaOH (40 per cent followed by 1 per cent), cooled, and mixed with one-third of its volume of strychnine molybdate reagent. The strychnine phosphomolybdate precipitate formed was centrifuged down after 10 minutes and treated similarly to the precipitate of the CP phosphate.

The various phosphate fractions, after being separated and made to volume in solution, were then ready for chemical and radioactivity analyses. The chemical analyses for phosphorus were carried out by the method of Dyer and Wrenshall (7), with small modifications worked out by Dr. S. B. Barker, formerly of this laboratory. The colorimeter measurements were made with an Evelyn photoelectric colorimeter, with a 660 m $\mu$  filter. Analyses on the IP phosphate fractions were carried out on aliquots containing 10 to 20  $\gamma$  of P; for the other phosphate fractions aliquots containing 3.5 to 7  $\gamma$  were used, since the strychnine present tended to cause clouding during the color development if over 7  $\gamma$  of P were present. The strychnine phosphomolybdate precipitates also gave a yellow color to their solutions, which, however, did not interfere with the measurements of the intensity of the ceruleomolybdate color.

The validity of the method employed for the separation of phosphate fractions in tissue was checked thoroughly as follows: (1) by comparison of the analyses by this method and by established methods for IP, CP, APP, and residual phosphate both in cardiac muscle extracts and in solutions of known composition; (2) by recovery analyses on cardiac muscle extracts with known amounts of added IP, CP, and APP. The purpose of this method was to permit the separation of the different phosphate fractions so that they could be analyzed individually both for concentration and for radioactivity. It is similar in part to the method of Sacks (8) who used ammonium molybdate rather than strychnine molybdate as a precipitating agent.

One difference between the method described and conventional methods is the more rapid completion of the hydrolyses of CP and APP. This is due to the higher concentration of acid employed for hydrolysis. The completeness of the APP hydrolysis after 5 minutes is evident from Fig. 1, which illustrates a typical experiment. The insignificant slope of the curve after 5 minutes also excludes the presence of appreciable amounts of hexose diphosphate (9).

No attempt has been made here to separate the individual components of the residual phosphate fraction. In a previous report (3), it was incorrectly assumed that in cardiac muscle 80 per cent of this fraction, exclusive of its adenylic acid, was hexose monophosphate. This was based on results

obtained with the Cori procedure for separating hexose monophosphate from skeletal muscle. The different results obtained with this procedure on cardiac and skeletal muscles were pointed out to us by Dr. G. Cori. At her suggestion the reducing power and P content of the hexose monophosphate from cardiac muscle were investigated in this laboratory with Dr. S. B. Barker. It was found that very little of this fraction is actually hexose monophosphate. Its exact composition has not, as yet, been determined.

*Radioactivity Measurements*—The radioactive phosphorus was kindly furnished us by Dr. John H. Lawrence of the Radiation Laboratory of the University of California. It was received in the form of a solution of  $\text{Na}_2\text{HPO}_4$  which was then converted to M/75 phosphate of pH 7.4 in isotonic NaCl solution. The amount of activity added per 25 cc. of medium for incubation was generally about 35 to 40 microcuries, or about 1,500,000

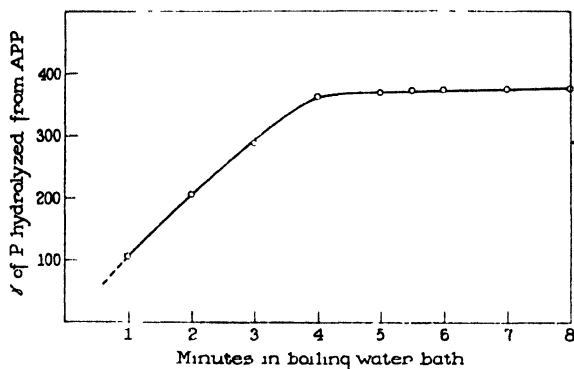


FIG. 1. Typical hydrolysis curve of adenylyl pyrophosphate in presence of acidified strychnine molybdate reagent.

counts per minute on our counter. This amount was usually contained in 0.5 to 2.5 cc. of the M/75 phosphate solution, the volume varying with the strength of the radioactivity.

The amount of activity used was too little to cause any injury to the tissue. Cardiac slices incubated with the usual amount of  $\text{P}^*\text{O}_4$  showed no significant differences from slices incubated without  $\text{P}^*\text{O}_4$  with respect to R.Q.,  $\text{QO}_2$ , and concentrations of different phosphate fractions. Slices incubated with as much as 3 times the usual amount of  $\text{P}^*\text{O}_4$  or with as little as one-tenth of it showed no significant differences in relative activities and concentrations of the phosphate fractions from slices incubated with the usual amount of  $\text{P}^*\text{O}_4$ .

One aspect of the preparation of the  $\text{P}^*\text{O}_4$  solution for use should be stressed. Any inorganic pyrophosphate contained in the  $\text{P}^*\text{O}_4$  sample should first be converted to inorganic orthophosphate. Most of the  $\text{P}^*\text{O}_4$  samples

received from the Radiation Laboratory of the University of California have contained small percentages of pyrophosphate along with the orthophosphate. If such samples are used in experiments such as those reported here, some inorganic pyrophosphate penetrates the tissue and eventually appears in the organic APP fraction. Although, as a contaminant, it does not add much to the total P concentration of this fraction, it raises its specific radioactivity disproportionately. This contamination with inorganic pyrophosphate was not appreciated until after the publication of the first radioactivity study from this laboratory (3), and as a consequence the values reported for the specific activity of the APP fraction were generally too high.

For all subsequent work, however, the  $P^*O_4$  samples were heated in a water bath for a sufficient period with HCl to convert any inorganic pyrophosphate present to orthophosphate. The amount of HCl used was adjusted so that the subsequent dilution and pH adjustment (to 7.4) of the  $P^*O_4$  sample with NaOH left a solution also isotonic with respect to NaCl.

The radioactivity measurements were made with a scale-of-four Geiger-Müller counter built by Dr. William Bale of the Department of Radiology, Strong Memorial Hospital, Rochester, New York. The counter tube was the "dipping" type designed by Bale *et al.* (10). The same solutions of the phosphate fractions used for P analyses were used for radioactivity analyses. Corrections for radioactivity decay were all made back to noon of the day of tissue incubation. Dilutions were made when necessary so that the solutions applied to the counter tube would not give more than 200 counts per minute. Trial experiments showed that for the same amount of  $P^*O_4$ , activity and concentration analyses gave the same results whether the  $P^*O_4$  solutions were made from precipitates of  $MgNH_4PO_4$ , strychnine phosphomolybdate, or ammonium phosphomolybdate.

A small correction was regularly made in calculating the activity and concentration of the CP fraction. It was based on the fact that about 1 per cent of IP is left behind by the  $MgNH_4PO_4$  precipitation and is later precipitated along with the CP phosphate as strychnine phosphomolybdate.

To facilitate comparison of results in any series of experiments reported here, specific radioactivities (counts per minute per unit weight of P) in each experiment are expressed as relative rather than absolute specific activities; that is, they are expressed as percentages of the absolute activity of one fraction taken as 100.

### Results

*Distribution of Inorganic Phosphate of Excised Cardiac Tissue between Extracellular and Intracellular Fluid*—By means of the washing technique described above, it has been found possible to separate the inorganic phos-

phate (IP) of cardiac tissue slices into an *easily diffusible fraction*, which could be readily washed out of the slices, and a *very poorly diffusible fraction*, which was retained despite prolonged washing. The easily diffusible fraction (hereafter called IP<sub>1</sub>) could be completely washed out of the tissue in 1 hour; the remaining poorly diffusible or "bound" fraction (hereafter called IP<sub>2</sub>) could not be decreased by more than a few per cent with further washing for an hour.

After a standard incubation in M/75 phosphate-Ringer's solution, about 30 to 40 per cent of the total IP of the tissue consisted of the easily diffusible IP<sub>1</sub> fraction. The specific radioactivities of IP<sub>1</sub> and IP<sub>2</sub> also proved to be

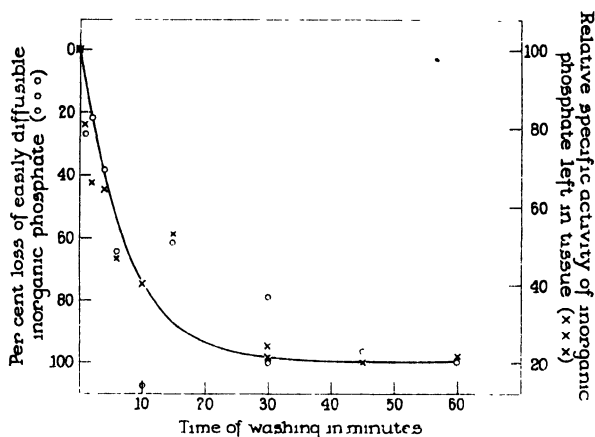


FIG. 2 Effect of washing in phosphate-free Ringer's solution on concentration and specific activity of inorganic phosphate of cardiac slices, incubated at 2° in M/75 phosphate for 30 minutes. Specific activities of IP left in tissue are given relative to the specific activity of the total IP in tissue before washing taken as 100. The curve is the theoretical diffusion curve of form  $C = C_0 e^{-Kt}$ .

quite different; that of IP<sub>1</sub> was always much greater than IP<sub>2</sub> even after several hours of incubation with P\*O<sub>4</sub>.

The effects of the washing procedure on the concentration and specific activity of the IP fractions of cardiac slices are illustrated in Fig. 2. Incubation with P\*O<sub>4</sub> was carried out at 2° for 30 minutes; in the subsequent washing changes of phosphate-free Ringer's solution were made after each withdrawal of tissue. The easily diffusible fraction, IP<sub>1</sub>, decreased rapidly at the beginning of washing and practically all of it had left the tissue within an hour. The decrease of the IP<sub>1</sub> concentration in the tissue with washing caused a simultaneous decrease in the specific activity of the IP fraction which remained. This specific activity reached a minimum level when all the IP<sub>1</sub> had been washed out. This level represents the specific activity of the poorly diffusible or "bound" fraction, IP<sub>2</sub>.

A second set of experiments was carried out, with the same technique but incubation of the tissues with  $P^*O_4$  at  $37.5^\circ$  to increase metabolic activity. During the washing process, changes of Ringer's solution were made every 15 minutes. The results of these experiments are given in Table I. Decreases were again observed in concentration and specific activity of the IP remaining in the tissue after the  $IP_1$  fraction was washed out. In Experiments 4 and 7 the washing procedure was prolonged beyond 60 minutes. In Experiment 4, washing for 75 minutes caused no significant change in either concentration or activity of the IP fraction from that observed at 60 minutes. In Experiment 7, however, prolongation of the washing for 90 minutes resulted in an increase in the IP concentration and a lowering of its specific activity as compared with the values at 60 minutes. If these dif-

TABLE I

*Effect of Washing on Concentration and Specific Activity of Inorganic Phosphate of Cardiac Slices after Incubation in Ringer-M/75  $P^*O_4$  at  $37.5^\circ$*

Experiment No	Length of incubation	Washing time	P before washing	Change in concentration on washing	Change in specific activity on washing
	<i>min</i>	<i>min</i>	<i>mg per cent</i>	<i>per cent</i>	<i>per cent</i>
1	30	60	35.7	-33	-58.4
2	30	60	31.6	-20	-54.5
3	30	60	31.4	-34	-46.0
4	30	60	32.5	-40	-67.3
		75	32.5	-40	-69.7
5	90	60	33.2	-29	-35.9
6	90	60	33.6	-42	-43.7
7	90	60	36.8	-37	-41.6
		90	36.8	-34	-47.6
Mean $\pm$ P.E.M			33.5 $\pm$ 0.52	-34.3 $\pm$ 1.73	

ferences are significant, they may have originated from the splitting of small amounts of tissue organic phosphates of low specific activity to IP during the prolongation of washing.

From the activity and concentration of the tissue IP before and after a period of washing, the specific activity of the IP washed out (that is,  $IP_1$ ) during that period was calculated, and compared with the specific activity of the IP of the incubation medium. The results of these experiments are given in Table II. The specific activities of the total IP of the unwashed tissue and of the  $IP_2$  fraction after washing were considerably lower than that of the IP of the incubation medium, whereas the specific activity of  $IP_1$  is generally within 10 per cent of it (except for one unusually low value of 56 per cent, probably the result of experimental error). These results indicate that after 30 minutes incubation with  $P^*O_4$  the specific activity of

extracellular IP ( $IP_1$ ) reached that of the medium IP; indeed, earlier experiments not reported here indicate that  $IP_1$  may attain the specific activity of the IP of the medium after as short an exposure to  $P^*O_4$  as 10 minutes at  $37.5^\circ$ . Apparently the easy diffusibility of  $IP_1$  permits it to come into rapid equilibrium with the IP of the medium.

These experiments (Table II) also serve to show the influence of temperature and the length of incubation on the specific activity of the  $IP_2$  fraction of the tissue. At  $37.5^\circ$ , about 4 times as much activity enters this fraction during a 30 minute incubation as at  $2^\circ$ . A 90 minute incubation at  $37.5^\circ$  causes a definite increase in specific activity over that of a 30 minute incubation period. The implications of these phenomena will be discussed later.

TABLE II

*Influence of Temperature and Duration of Incubation in Ringer-M/75  $P^*O_4$  on Relative Specific Activities of Various Inorganic Phosphate Fractions*

The specific activity of IP of the medium is taken as 100.

Length of incubation	Temperature of incubation	Relative specific activities		
		Total IP of unwashed tissue	Diffusible IP ( $IP_1$ )	Non-diffusible IP ( $IP_2$ )
min	$^\circ C$			
30	2	25	104	5
30	2	25	56	5
30	2	28	95	2
30	37.5	46	91	19
30	37.5	56	107	17
30	37.5	55	112	24
90	37.5	52	94	33
90	37.5	65	104	37
90	37.5	51	94	26

*Effect of Washing Procedure on Concentration and Specific Activities of Creatine Phosphate and Adenyl Pyrophosphate Fractions*—In addition to IP measurements in the experiments listed in Table I, the concentrations and specific activities of the creatine phosphate (CP) and adenyl pyrophosphate (APP) fractions were also determined before and after the washing process. The results are summarized in Table III. Certain differences were noted between the behavior of the IP, CP, and APP fractions on washing. As has already been noted and shown in Table I, the total inorganic fraction (IP) decreased markedly in both total concentration and specific activity. The CP phosphate decreased to about the same extent in concentration, but its specific activity did not change significantly from the value prior to washing. Unlike the IP, therefore, the CP in the tissue appears to be homogeneous as to specific activity. It still remains



to be determined whether the CP lost in the washing procedure is hydrolyzed and diffuses out of the tissues or whether it diffuses out as such.

The APP phosphate is decreased slightly on the average during the washing procedure both in concentration and specific activity. In individual experiments, however, these changes are often too small to be of significance. It is suggested that such significant decreases as occur are due to the splitting of small amounts of the terminal labile phosphate group of the adenosine triphosphate of the APP fraction during the washing. How this could cause a decrease in the specific activity as well as the concentration of this fraction will become apparent later in this paper.

TABLE III

*Effect of Washing for 1 Hour on Concentration and Specific Activity of Creatine Phosphate and Labile Phosphate of Adenyl Pyrophosphate of Cardiac Slices after Incubation in Ringer-M/75 P\*O<sub>4</sub> at 37.5°*

Experiment No	Length of incubation	CP			APP		
		P before washing	Change in concentration on washing	Change in specific activity on washing	P before washing	Change in concentration on washing	Change in specific activity on washing
	min	mg per cent	per cent	per cent	mg per cent	per cent	per cent
1	30	16.7	-51	-2.5	11.1	-0.2	-11.7
2	30	12.7	-38	-4.8	7.3	+7.9	-11.8
3	30	19.3	-28	-3.0	11.4	-6.3	+1.8
4	30	14.2	-24	+8.4	9.1	-3.9	+2.9
5	90	15.1	-42	+2.1	11.6	-12.1	-5.3
6	90	14.2	-20	+0.6	9.5	+2.6	-7.3
7	90	18.8	-24	-2.0	11.1	-3.1	-8.5
Mean ± P.E.M.		15.9 ±0.63	-32 ±2.92	-0.17 ±0.11	10.2 ±0.40	-2.2 ±0.16	-4.4 ±0.16

The data obtained in these experiments were then considered in terms of the relationship between the specific activities of CP and APP and those of the IP fractions, since this relationship might give indication of the amount and direction of phosphate turnover from IP through the organic phosphate fractions. The data showing this relationship for two typical experiments have been assembled in Table IV, and show again the effect of washing on concentration and specific activities of the phosphate fractions noted above. The significant new correlation is that in these experiments in which incubation with P\*O<sub>4</sub> was carried out at 37.5°, the specific activity of the CP phosphate is equal to that of the IP remaining in the tissue after washing; that is, it is equal to that of the intracellular IP<sub>2</sub>. Table V, which includes all the experiments of this group, shows the consistency

of this equality. The values are all expressed relative to the specific activities of  $IP_2$ .

TABLE IV

*Effect of Washing on Concentrations and Specific Activities of Inorganic Phosphate, Creatine Phosphate, and Adenyl Pyrophosphate after Incubation in Ringer-M/75  $P^*O_4$  at 37.5°*

The specific activity of IP of the medium is taken as 100.

Experiment No.	Time of washing	IP		CP		APP	
		Specific activity	Concentration	Specific activity	Concentration	Specific activity	Concentration
	<i>min</i>		<i>mg. per cent P</i>		<i>mg per cent P</i>		<i>mg. per cent P</i>
5	0	52	33.2	32	15.1	27	11.6
	60	33	23.5	33	8.8	25	10.2
6	0	65	33.6	37	14.2	29	9.5
	60	37	19.4	37	11.4	27	9.7

TABLE V

*Relative Specific Activities of Various Acid-Soluble Organic Phosphate Fractions after Incubation in Ringer-M/75  $P^*O_4$  at 37.5°*

The intracellular IP is taken as 100

Experiment No	Length of incubation	Relative specific activities	
		CP	Labile phosphate of APP
	<i>min</i>		
1	30	103	78
2	30	97	71
3	30	98	63
4	30	101	69
Average		100.3	70.3
5	90	97	82
6	90	101	77
7	90	97	75
Average.		98.3	78.0

The average relative specific activity for residual P, as found in earlier experiments is about 25.

The relative specific activity of the labile phosphate of the APP was, however, always less than that of the two other fractions. After 30 minutes incubation, the activity of the labile phosphate was about 70 per cent, and after 90 minutes incubation about 80 per cent, of the respective  $IP_2$  values. This labile pyrophosphate fraction in excised cardiac muscle

has been shown to consist of a mixture of labile phosphate from both adenosine triphosphate and adenosine diphosphate (11). Certain unpublished observations of our own confirm this earlier finding. On finding that the specific activity of the labile phosphate of the APP in experiments such as are described here was lower than the specific activities of the CP and the intracellular inorganic phosphate, we considered the possibility that the total pyrophosphate fraction contained phosphorus of both high and low specific activities. In particular, it was thought that perhaps the terminal labile phosphate of the adenosine triphosphate present in the pyrophosphate mixture might have the same specific activity as the CP, since, according to modern concepts, this terminal phosphate is in a rapid equilibrium exchange reaction with the phosphate of CP. The problem therefore presented itself of determining separately the specific activity of the terminal phosphate of adenosine triphosphate.

*Determination of Specific Activity of Terminal Labile Phosphate of Adenosine Triphosphate Present in Adenyl Pyrophosphate Fraction*—The method used for determining the specific activity of the terminal phosphate group of adenosine triphosphate in the pyrophosphate fraction was based on the finding of Lohmann (12) that crayfish muscle, washed free of magnesium ions, was able to split off the terminal but not the second phosphate of adenosine triphosphate. An enzymatic muscle suspension containing 30 gm. in 150 cc. was made from the claw muscles of a lobster according to Lohmann's directions. This suspension, however, was found to split both labile phosphates from adenosine triphosphate. Apparently further washing was necessary to free it of magnesium. The following procedure resulted in a successful preparation. The lobster muscle contained in 2 cc. of the above suspension was washed three times with 10 cc. portions of 0.45 per cent KCl and then with 10 cc. of distilled water, with thorough mixing of muscle and washing fluid between centrifugations. After the final centrifugation, the muscle was resuspended to a volume of 2 cc. and 10 cc. of the solution containing the adenosine triphosphate added. The mixture was allowed to incubate, with stirring, at room temperature and the muscle then centrifuged down. Control analyses with adenosine triphosphate<sup>1</sup> showed that all of the terminal phosphate (about 100  $\gamma$  of P) was split off in 5 minutes and that further incubation up to 20 minutes caused no liberation of the second labile phosphate. The second labile phosphate, however, was liberated completely if magnesium was added to the lobster muscle preparation.

Three radioactivity experiments with tissue were then carried out by

<sup>1</sup> The barium salt of adenosine triphosphate was kindly furnished by Dr. S. P. Colowick of Washington University School of Medicine, and was converted by us into a solution of sodium salt.

means of this method to determine the specific activity of the terminal phosphate of adenosine triphosphate in the APP fraction. About 15 gm. of cardiac tissue were employed in each experiment. They were incubated in the usual fashion for 30 minutes with radioactive phosphorus, washed for 60 minutes with change of Ringer's solution every 15 minutes, and extracted with 5 per cent trichloroacetic acid. In each experiment one pool was made of the acid extracts of the washed tissues. On two 10 cc. aliquots of the pooled extract, determinations of  $IP_2$  (intracellular IP), CP, and APP, were made in the usual fashion. A larger portion of the pooled extract (40 cc. in one experiment, 105 cc. in the other two) was put through the Cori procedure (6) for precipitation of the barium salts of the IP and APP fractions. The precipitation was carried out twice, followed each time by washing with water and redissolving in a small quantity of 0.01 M HCl. The barium was removed from the final solution with a sodium sulfate solution and the acid neutralized with dilute NaOH. The final volume was made up to about half of the original volume of the acid extract.

From the resulting solution (containing the sodium salts of IP, adenosine diphosphate, and adenosine triphosphate), 10 cc. aliquots were taken for determining the change in concentration and activity of the APP fraction on incubation with the washed lobster muscle preparation. They were incubated for 5 or 10 minutes with washed lobster muscle, centrifuged, and the supernatant fluids fractionated for IP and APP labile phosphate. The aliquots used as controls were fractionated directly for  $IP^2$  and APP labile phosphate, except in one experiment when they were first incubated with a heat-inactivated lobster muscle preparation.

The results of the analyses for the concentration and the radioactivity of the labile phosphate of the APP fraction are assembled in Table VI. From the decrease in concentration and specific activity of the APP fraction after this procedure, the specific activities of the terminal phosphate of the adenosine triphosphate, split off during incubation, could be calculated. These activities are given in Column *e*. In Column *f*, the specific activities of CP, of the terminal phosphate of the adenosine triphosphate of the APP fraction, and of the unliberated labile phosphate of the APP are all given relative to that of  $IP_2$  taken as 100. It will be noted that the specific activities of the phosphates of  $IP_2$  and CP and the terminal phosphate of the adenosine triphosphate in the APP fraction are now identical.

It is also apparent from Table VI that the pyrophosphate fraction

\* The precipitation of the IP here differed from the precipitation from the trichloroacetic acid extracts in that 0.3 cc. of concentrated  $NH_4OH$ , 1 cc. of 3 N  $NH_4NO_3$ , and 1 cc. of 15 per cent  $Mg(NO_3)_2 \cdot 6H_2O$  were added to each 10 cc. aliquot.

obtained from cardiac slices contains considerable labile phosphate in addition to that which can be accounted for by the adenosine triphosphate present. Undoubtedly a large part of this extra labile phosphate is present in adenosine diphosphate, as was found in excised heart muscle by Lohmann and Schuster (11). One additional observation which bears out this contention may be noted. An aliquot from the pyrophosphate of Experiment 10, Table VI, was incubated with lobster muscle plus 22 mg. of  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  to permit the liberation of labile phosphate of adenosine diphosphate originally present or formed during incubation.<sup>3</sup> By this

TABLE VI

*Experiments to Determine Specific Activity of Terminal Labile Phosphate of Adenosine Triphosphate in Adenyl Pyrophosphate (APP) Fraction of Cardiac Slices after 30 Minutes Incubation with  $\text{P}^3\text{O}_4$  at 37.5°*

Experiment No.	Specific activity of intra-cellular IP ( $\text{IP}_2$ )	Specific activity of CP	Labile P from APP in 10 cc solution before incubation with washed lobster muscle			Labile P from APP in 10 cc solution after incubation with washed lobster muscle*			Calculated specific activity of terminal P of APP† (e)	Relative specific activities (f)			
	(a)	(b)	(c)			(d)			counts per min per $\gamma$	$\text{IP}_2$	CP	Terminal P of APP	Remaining labile P of APP
	counts per min per $\gamma$	counts per min per $\gamma$	counts per min	$\gamma$ of P	counts per min per $\gamma$	counts per min	$\gamma$ of P	counts per min per $\gamma$					
8‡	17.2	16.9	673	66 0	10 2	428	51 5	8 3	16.9	100	98	98	49 2
9	27.5	28.2	2680	143 0	18 7	1354	96 5	14 0	27.9	100	102	101	49 6
10§	16.8	17.3	1310	107 0	12 2	588	64 0	9 2	16.8	100	103	100	53 1

\* In Experiment 8 the incubation was for 5 minutes and in Experiments 9 and 10 for 10 minutes

† Sample calculation (Experiment 8) for Column e: specific activity of terminal P of APP =  $\frac{(\text{counts per min. from Column c}) - (\text{counts per min. from Column d})}{(\gamma \text{ of P from Column c}) - (\gamma \text{ of P from Column d})}$

$$\frac{673 - 428}{66.0 - 51.5} = 16.9.$$

$$\frac{66.0 - 51.5}{143.0 - 107.0} = 16.9.$$

‡ Duplicate aliquots were not used in this experiment because of lack of material.

§ Controls here were incubated with a heat-inactivated lobster muscle suspension.

procedure 90 per cent of the total labile phosphate of the pyrophosphate fraction was liberated as IP in 45 minutes.

*Effect of Incubation at 2° on Distribution of Radioactive Phosphate in Acid-Soluble Phosphate Fractions of Cardiac Slices*—Incubations at 2° were carried out to see whether the reduction in metabolism brought about

<sup>3</sup> Myosin may be the phosphatase for the terminal phosphate of adenosine triphosphate (13). This phosphatase, acting in conjunction with myokinase plus its coenzyme, magnesium, appears to participate in the reaction by which the labile phosphate of adenosine diphosphate is liberated (14).

at this low temperature might affect the relative specific activities of the acid-soluble phosphate compounds of cardiac slices. The results of such incubations are given in Table VII. In this table, where all specific activities are given relative to those of the  $IP_2$  taken as 100, it can be seen that the specific activities of the  $IP_2$  are significantly higher than those of the organic phosphate fractions. The specific activity of CP is less than 50 per cent of that of the  $IP_2$ . The specific activity of the pyrophosphate fraction is still lower, and, as in the experiments carried out at  $37.5^\circ$ , is also about 70 per cent of that of the CP. These results will be discussed later with reference to the question as to whether extracellular inorganic phosphate exchanges *directly* with organic phosphates, or only after first entering the cell as inorganic phosphate.

TABLE VII

*Effect of 30 Minute Incubation at  $2^\circ$  in Ringer-m/75  $P^*O_4$  on Specific Activities of Intracellular Inorganic Phosphate and Organic Phosphate Fractions of Cardiac Slices*  
The specific activity of intracellular IP is taken as 100

CP	Labile P of APP	Residual organic phosphate
47	35	5
41	29	5
45	34	4

## DISCUSSION

*Partition of Tissue Inorganic Phosphate*—By means of the washing technique following incubation in m/75  $PO_4$  the tissue IP was found to consist of a readily diffusible fraction, which comprised about 30 to 40 per cent of the total IP, and a larger fraction which was retained in the tissue despite prolonged washing. On the basis of the difference in diffusibility, the first fraction may be regarded as extracellular, the second as intracellular IP. The failure to reduce the intracellular phosphate, even on prolonged washing with phosphate-free Ringer's solution, is in accord with the finding of Eggleston (15) on the retention of intracellular phosphate of frog muscle in phosphate-free Ringer's solution. A calculation of the amount of extracellular fluid in excised cardiac slices, based on the average values from Table I, and a P concentration of the usual value of 40 mg. per cent in the medium, gave a value of about 29 per cent. This is somewhat higher than the value of 23.2 per cent found by Wood (16) for the ventricular muscle of dogs; but it is quite likely that the slicing of the ventricle in our procedure adds somewhat to the extracellular space.

*Rate of Penetration of  $P^*O_4$  into Intracellular Space*—Apparently the penetration of  $P^*O_4$  from the extra- into the intracellular space is not a matter of simple diffusion, since the penetration is 5 to 10 times as rapid at

37.5° as at 2°. It also does not seem to be a simple function of the total metabolism, for the rate of metabolism of cardiac slices at 37.5° is much greater than 10 times that at 2°. The rate of penetration of phosphate from the extra- into the intracellular spaces at 37.5° can be calculated from the specific activity of extracellular IP and the increase in *total* intracellular radioactivity with time. The average value was found to be about 1.7  $\gamma$  of P per minute per gm. of tissue. This is a much higher rate of penetration than was found for phosphate entering the intracellular spaces of the rabbit gastrocnemius *in vivo* (17), but it is of about the same order of magnitude as that of phosphate entering the intracellular space of the rat heart, as can be roughly calculated from the data of Manery and Bale (18). The rate of exchange of IP from the intra- to the extracellular phase, that is from IP<sub>2</sub> to IP<sub>1</sub>, may be assumed from our experiments to be the same as that from IP<sub>1</sub> to IP<sub>2</sub>, since, in the course of a 1 or 2 hour incubation of cardiac slices, the *concentration* of the total IP and the organic phosphate in the tissue remains comparatively constant.

*Rate and Direction of Phosphate Uptake by Organic Phosphate Fractions—*

Before these data are evaluated, the results of previous investigations may be briefly reviewed. Korzybski and Parnas (19) reported a rapid turnover of phosphate through the APP and CP of rabbit muscle. Sacks (8) found the specific activity of the total labile phosphate of the pyrophosphate fraction in heart muscle *in vivo* very close to that of the CP after P\*O<sub>4</sub> injection. Hevesy and Rebbe (20) reported that the specific activity of the labile phosphate of the pyrophosphate fraction was equal to that of the CP in frog gastrocnemius 3 hours after injection. The discrepancy between these findings and those reported here may be due to the fact that with cardiac slices *in vitro* a certain part of the pyrophosphate fraction may not be involved in a rapid metabolic turnover whereas all of this fraction may be involved in a rapid metabolic turnover *in vivo*.

The data obtained in the present study indicate that the *intracellular* IP rather than the total tissue IP is the direct source for the supply of phosphate to APP and CP. No regular relationship can be established between the specific activities of the organic phosphates and the total tissue IP, whereas a state of equality was uniformly observed between the specific activities of intracellular IP, of CP, and of the terminal phosphate of APP. As will be shown, the existence of such an equality provides very strong support for regarding the intracellular IP rather than the total tissue IP as the primary donator.

Sacks and Altshuler (21) reported that they found a difference in the specific activities of the intracellular IP and of the CP both in skeletal and cardiac muscle of cats after the intravenous injection of radioactive phosphate. The disagreement between their results and ours may lie

in their calculation of the activity of the intracellular phosphate. They have to make the assumptions that an equilibrium exists both in concentration and radioactivity between the IP of the plasma and the IP of the extracellular fluid, and that the muscle under experimentation has a certain definite extracellular space. The extreme variations which they obtained in the calculated values for the intracellular IP in similar experiments, suggest the unreliability of these assumptions. Since their conclusions as to the rôle of phosphate transfer in muscle are based on results which are frequently the averages of widely discrepant values, one may reasonably defer accepting their further inference that they serve to discredit the Meyerhof theory, until more consistent experimental data are provided. Sacks and Altshuler's conclusion that extracellular IP and intracellular IP exchange through CP and APP, which act as phosphate carriers, is not in agreement with our findings, shortly to be discussed, which indicate that the extracellular IP exchanges directly with the intracellular IP.

The experiments of Bollman and Flock (22) on the effects of contraction *in vivo* on the distribution of  $P^*O_4$  among the acid-soluble phosphate fractions of rat leg muscle are also pertinent to this discussion. They found that the uptake of  $P^*O_4$  by CP and APP respectively was equal in the exercised and resting limb. Nor was there a higher specific activity in the CP and APP fractions of the exercised than of the resting limb following their resynthesis during the recovery period. These findings would be difficult to explain were the *total* tissue IP, with a specific activity several times greater than that of CP and APP, the direct donator of phosphate for the resynthesis of CP and APP. However, these results would be satisfactorily explained by the concepts developed in this paper, since the phosphate for the resynthesis of CP and APP after exercise would be derived from intracellular IP of equal specific activity; therefore no increase of the specific activity of these fractions on resynthesis would be anticipated. The similarity in both resting and exercised muscle between the specific activities of these phosphate fractions would only require the assumption that the exercise did not influence the rate of exchange between extra- and intracellular IP.

There remains for consideration the problem of how the phosphate of CP and the terminal phosphate of the adenosine triphosphate attain the same specific activity as that of the intracellular IP in the brief course of a 30 minute incubation at 37.5°.

The possibility was considered that the phosphorylation of the labile organic phosphate compounds was a means of transporting phosphate in the exchange,  $IP_1 \rightleftharpoons IP_2$ , and that the specific activity of  $IP_2$  was therefore limited by that of the organic phosphate. This hypothesis was discarded for several reasons, the main one being that at 2° the specific activity of



the intracellular IP rose to a much higher value than that of any of the organic phosphate fractions during a 30 minute incubation (Table VII). This provides direct evidence that the extracellular IP exchanges directly with intracellular IP without the mediation of an organic phosphate carrier, as postulated by Sacks.

A second possibility was that the intracellular IP might not exist as free IP in the living tissue. Both the terminal phosphate of APP and that of CP are high energy phosphates which can exchange rapidly in equilibrium. This rapid equilibrium exchange could account for the equality of the specific activity of CP and the terminal phosphate of APP. Were the intracellular IP bound *in vivo* to some substance by a high energy bond, it might also be in rapid equilibrium exchange with CP and APP. This bond would have to be of such a character as to split during the preparation of the trichloroacetic acid extract of the tissue. This hypothesis cannot at the moment be entirely disproved but seems unlikely if one accepts the current ideas concerning energy relations involved in phosphorylation coupled with oxidation. Some evidence against it may reside in the finding that HCl and H<sub>2</sub>SO<sub>4</sub> extracts of cardiac muscle gave the same results as trichloroacetic acid extracts.

The hypothesis may now be discussed which appears to provide the best present explanation of the specific activities demonstrated by this study. It has been shown by a number of workers that phosphorylations are coupled with the oxidative steps in the combustion of carbohydrates (for reviews see (23) and (24)) and that each phosphorylation first goes through adenosine triphosphate. Colowick *et al.* (25) have demonstrated, in heart muscle extracts, as many as twelve phosphorylations per molecule of glucose oxidized, or one phosphorylation for each atom of oxygen consumed. Ochoa (26) using brain dispersions and Belitser and Tsibakova (27) using minced heart muscle found from two to three phosphorylations per atom of oxygen consumed. These experiments involved the oxidation of glucose, of degradation products of glucose, or of 4-carbon acids. In this connection earlier experiments from this laboratory (3) may be cited indicating that the oxidation of *fat* may also be coupled with phosphorylation, since the phosphorylation of APP and CP occurs to the same extent in cardiac slices with a low R.Q. (around 0.75) and with an R.Q. close to unity.

On the basis of our experimental data, a phosphorylation rate for cardiac muscle slices may be calculated, assuming two phosphorylations through the terminal group of APP for every atom of oxygen consumed. The oxygen consumption of cardiac tissues from a normal well fed dog is about 1.0 cc. per moist gm. per hour. This is equivalent to 1/22,400 mole or 44.6 micromoles of oxygen per hour, which would cause 178.4

micromoles of phosphorylation per hour. This would represent a turnover in terms of P passing from intracellular IP ( $IP_2$ ) to APP of about 92  $\gamma$  per gm. of tissue per minute. Since the tissue is in a relatively steady state during these experiments, the rate of P going from APP back to  $IP_2$  is equal to that from  $IP_2$  to APP.

The phosphate cycles postulated as occurring in cardiac muscle are graphically represented in Fig. 3. The terminal phosphate of APP and the phosphate of CP are shown in a rapid equilibrium exchange which would always keep them at the same specific radioactivity in experiments with  $P^*O_4$ . Together they may be considered a pool of labile organic phosphate through which passes the phosphate from  $IP_2$ . This pool contains an average of about 200  $\gamma$  of P per gm. of sliced cardiac muscle, so that the rate of turnover of 92  $\gamma$  per minute per gm. calculated above would mean a virtually complete turnover through it every 2 minutes.

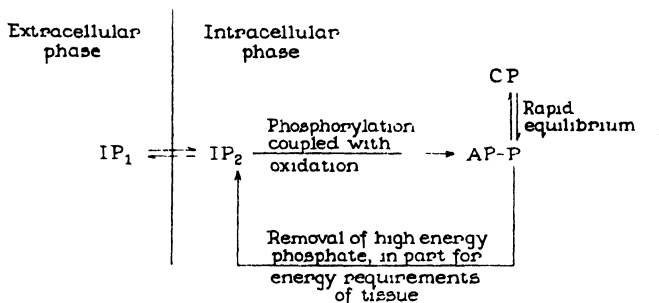


FIG. 3 Postulated cycle of phosphate exchange in cardiac muscle slices IP = inorganic phosphate, APP = adenylyl pyrophosphate, CP = creatine phosphate

This rapid turnover of phosphate would be an efficient mixing cycle for the phosphate of CP, the terminal phosphate of APP, and of  $IP_2$ . Since the  $IP_1 \rightleftharpoons IP_2$  exchange is very slow in comparison (1.7  $\gamma$  per minute per gm.), this rapid turnover might be expected to bring the specific activities of CP and the terminal phosphate of APP to a value insignificantly different from that of  $IP_2$ , in the course of a 30 minute incubation with  $P^*O_4$ .<sup>4</sup>

<sup>4</sup> It is also possible to show mathematically that the specific activity of the terminal P of APP and the P of CP could practically attain that of  $IP_2$  after 30 minutes of incubation with  $P^*O_4$ , if the cycle shown in Fig. 3 exists. The terminal P of APP and the P of CP will be assumed to constitute together a labile organic phosphate pool (OP). To simplify matters it will also be assumed that all the terminal phosphate radicals removed from APP go directly to IP molecules. This simplification neglects the passage of phosphate radicals from APP through other molecules such as hexose diphosphate before becoming IP molecules. (In resting muscle the proportion of phosphate radicals split from APP not going directly to IP molecules is probably

In the above scheme the specific activity of the second labile phosphate of adenosine triphosphate has not been discussed, as experiments have not yet been performed to obtain its value. Such experiments are contemplated and will involve an isolation of the adenosine triphosphate of the pyrophosphate fraction. However, the hypothesis presented here to explain our results will remain fundamentally unaltered, whether the second labile phosphate is found to have a specific activity equal to or lower than that of the terminal labile phosphate.

Apparently, therefore, the equalities observed in this study between the specific activities of  $IP_2$ , CP, and the terminal phosphate of APP after incubation with  $P^*O_4$  can be adequately explained by resorting to the modern concepts of the relationship between phosphorylations and oxidations in tissues. It is encouraging to find that studies on living tissue *in vitro* can provide evidence in support of complex reactions for whose study non-viable extracts are usually employed.

small.) By neglecting the indirect passage, an extra diluting effect on the activity of the phosphate going into  $IP_2$  is removed, so that the calculated specific activity of OP relative to  $IP_2$  will probably be somewhat lowered.

The increase of activity in the OP fraction with time is given by

$$dx/dt = K(K'\alpha_{IP_1}t - x - y)/C_{IP_2} - Kx/C_{OP} \quad (1)$$

where  $x$  is the activity of OP;  $K$  is the rate of transfer for  $IP_2 \rightarrow OP$  and also for  $OP \rightarrow IP_2$  (reactions of equal rate at steady state);  $K'$  is the rate of transfer for  $IP_1 \rightarrow IP_2$  and also for  $IP_2 \rightarrow IP_1$ ;  $\alpha_{IP_1}$  is the specific activity of  $IP_1$ ;  $t$  is the time;  $y$  is the activity which returns from the intracellular to the extracellular space by  $IP_2 \rightarrow IP_1$ ; and  $C_{IP_2}$  and  $C_{OP}$  are respective concentrations of  $IP_2$  and OP. During the course of a 30 minute incubation,  $K$ ,  $K'$ ,  $\alpha_{IP_1}$ ,  $C_{IP_2}$ , and  $C_{OP}$  all stay practically constant. Also

$$y = K' \int_0^t \alpha_{IP_1} dt \quad (2)$$

where  $\alpha_{IP_2}$  is the specific activity of  $IP_2$ . We found in earlier experiments that  $\alpha_{IP_2}$  increases almost linearly over 30 minutes incubation, giving approximately

$$\alpha_{IP_2} = 0.005 \alpha_{IP_1} t \quad (3)$$

where  $t$  is the time in minutes. Substituting Equation 3 in Equation 2 and Equation 2 in Equation 1 and arbitrarily setting  $\alpha_{IP_1}$  equal to unity, we get

$$dx/dt = K[K't - x - K'(0.0025t^2)]/C_{IP_2} - Kx/C_{OP} \quad (4)$$

or

$$\frac{dx}{dt} + K \left( \frac{C_{IP_2} + C_{OP}}{C_{IP_2} C_{OP}} \right) x = \frac{KK'}{C_{IP_2}} (t - 0.0025t^2) \quad (5)$$

Equation 5 may be integrated to give

$$x = \left( \frac{a}{b^2} + \frac{0.005a}{b^3} \right) (bt + e^{-bt} - 1) - \frac{0.0025at^2}{b} \quad (6)$$

where  $a$  is  $KK'/C_{IP_2}$  and  $b$  is  $K(C_{IP_2} + C_{OP})/C_{IP_2}C_{OP}$ .

## SUMMARY

1. Results are reported on the uptake of  $P^*O_4$  by the inorganic phosphate, creatine phosphate, and adenylyl pyrophosphate fractions of dog cardiac muscle slices exposed *in vitro* to  $P^*O_4$ .

2. The total tissue inorganic phosphate was found to consist of two fractions, a readily diffusible and a poorly diffusible or "bound" fraction. The former is considered extracellular, the latter, intracellular.

3. The extracellular phosphate came into rapid equilibrium with the  $P^*O_4$  of the medium.

4. The specific activity of the intracellular phosphate was always considerably less than that of the extracellular phosphate and was dependent in part on the extent of metabolism.

5. Results of experiments at  $2^\circ$  indicated that extracellular inorganic phosphate exchanged directly with intracellular inorganic phosphate without the mediation of an organic phosphate carrier.

6. The specific activity of the creatine phosphate after 30 to 90 minutes incubation with  $P^*O_4$  at  $37.5^\circ$  was equal to that of the intracellular inorganic phosphate, whereas the specific activity of the labile phosphates of the adenylyl pyrophosphate fraction was 70 to 80 per cent of it.

Now in 1 gm of cardiac slices  $C_{IP_2}$  and  $C_{OP}$  are about 200  $\gamma$  each. The value for  $K$ , based on two phosphorylations per atom of oxygen consumed, has already been shown to be about 92  $\gamma$  per minute, and that for  $K'$ , based on experimental results, to be about 1.7  $\gamma$  per minute. This makes  $a$  equal to 0.782 and  $b$  equal to 0.92. Then at the end of 30 minutes incubation:

$$x = \left( \frac{0.782}{(0.92)^2} + \frac{0.005 (0.782)}{(0.92)^2} \right) (0.92 (30) + e^{-0.92(30)} - 1) - \frac{0.0025(0.782) (30)^2}{0.92} = 22.80 \quad (7)$$

or the specific activity of OP after 30 minutes is

$$\alpha_{OP} = x/C_{OP} = 22.80/200 = 0.119 \quad (8)$$

The specific activity of  $IP_2$  after 30 minutes is

$$\alpha_{IP_2} = (K' \alpha_{IP_1} t - x - y)/C_{IP_2} \quad (9)$$

or

$$\alpha_{IP_2} = (1.7(30) - 22.80 - 3.33)/200 = 0.124 \quad (10)$$

Thus, the calculation gives specific activities of the CP and the terminal P of APP only slightly less (insignificantly less by our methods of analysis) than that of the  $IP_2$  after 30 minutes of incubation. This is so even in our simplified treatment, in which we assume that all of the terminal phosphate radicals leaving APP go directly to  $IP_2$ .

7. However, the adenylyl pyrophosphate fraction was found to be composed only in part of adenosine triphosphate. Liberation of the terminal phosphate of the adenosine triphosphate by a magnesium-free lobster muscle enzyme preparation showed its terminal phosphate to have the same specific activity as that of creatine phosphate and intracellular inorganic phosphate. The portion of the adenylyl pyrophosphate fraction not consisting of adenosine triphosphate may be in large part adenosine diphosphate.

8. The equality in the specific activities of the intracellular inorganic phosphate, creatine phosphate, and terminal phosphate of adenosine triphosphate of excised cardiac tissue incubated with  $P^*O_4$  is in accord with modern concepts as to the coupling of oxidation and phosphorylation in tissue.

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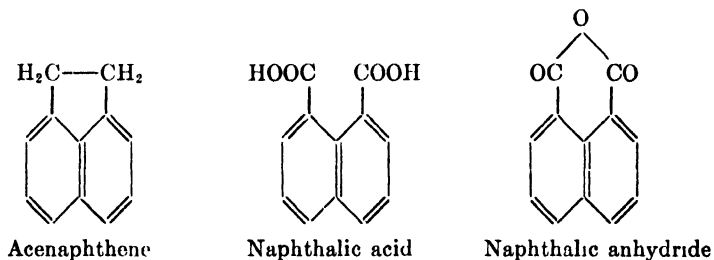
# THE METABOLISM OF ACENAPHTHENE IN THE RAT

By L. H. CHANG\* AND LESLIE YOUNG

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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It is known that the administration of naphthalene to animals is followed by the excretion in the urine of a compound which yields naphthalene on acidification (1). Anthracene (2) and phenanthrene<sup>1</sup> when administered to rats and rabbits give rise to the excretion of compounds which yield anthracene and phenanthrene respectively when decomposed by acid. The authors recently reported the results of a study of the excretion of acid-decomposable hydrocarbon precursors by the rat following the administration of various polycyclic hydrocarbons including acenaphthene (3). In this work no evidence was obtained of the liberation of acenaphthene on acidification of the urine of rats dosed with this hydrocarbon. From the acidified urine of these animals, however, a crystalline compound, m.p. 269°, was obtained.<sup>2</sup> The purpose of the present paper is to describe the isolation of this compound and its identification as naphthalic anhydride; *i.e.*, the anhydride of naphthalene-1,8-dicarboxylic acid.



The isolation of naphthalic acid or its anhydride from urine following the administration of acenaphthene to animals has not hitherto been reported. The present finding is of interest inasmuch as it indicates that fission of the 5-membered carbon ring of acenaphthene can occur in the animal body.

## EXPERIMENTAL

Acenaphthene was administered to male white rats, each of which weighed approximately 300 gm. The animals were housed in metabolism cages which permitted the collection of urine separate from the feces. The

\* Sino-British Fund Fellow. Present address, Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut.

<sup>1</sup> Young, L., and Britton, A., unpublished observations.

<sup>2</sup> Unless otherwise stated, the melting points recorded herein are uncorrected.

feces were inaccessible to the rats. The urine was collected daily, filtered through glass wool, and stored in the refrigerator until used. In some experiments the animals were fed in separate cages for two 1 hour periods daily on the stock colony diet<sup>3</sup> to which 1 per cent of acenaphthene had been added. Water was available to the rats at all times. In other experiments the rats received food and water *ad libitum* in the metabolism cages and on alternate days were each dosed by stomach tube with 1 ml. of a fine suspension of 0.1 gm. of acenaphthene in dilute starch solution. In order to minimize the scattering of food in these experiments the stock colony diet was made into a stiff dough by mixing with an equal amount of 5 per cent starch solution.

The isolation of the compound, m.p. 269°, together with its properties and the steps taken to identify it, is described below.

*Isolation*—The compound was obtained from urine by several different procedures, two of which are described below.

The urine of rats dosed with acenaphthene was made alkaline to litmus by the addition of dilute NaOH solution and was then extracted twice with ether. The urine was then made acid to Congo red by the addition of HCl and allowed to stand for an hour. The urine together with the precipitate which formed on acidification was extracted with four portions of chloroform, each of which was equal in volume to that of the urine. The chloroform extracts were combined, concentrated to a small volume by distillation on a water bath, and finally evaporated to dryness under reduced pressure. The residue was treated with hot ethanol and the solution was filtered rapidly. The crystalline precipitate which separated from the alcoholic solution on cooling was filtered and washed with a small volume of warm ethanol. The product melted at 268°. It was dissolved in warm N NaOH solution, decolorizing charcoal was added, and the solution was warmed and filtered. The precipitate which formed on acidification of the filtrate with HCl was filtered. It was dried over phosphorus pentoxide *in vacuo*, and after being crystallized twice from ethanol it was obtained as pale yellow needles, m.p. 269°. The amount of the compound obtained from the urine by the above procedure was small. In an experiment in which three rats were each dosed on alternate days with 1 ml. of a suspension of 0.1 gm. of acenaphthene in dilute starch solution until they had received a total dosage of 1.8 gm. of hydrocarbon, the amount of pure compound isolated from the urine was 0.060 gm. From the urine of six rats which received a diet containing 1 per cent of acenaphthene until they had ingested 4.1 gm. of the hydrocarbon, 0.215 gm. of the compound was isolated.

<sup>3</sup> The composition of the stock diet was as follows: ground corn 50, dried milk 25, linseed meal 15, casein 3.5, alfalfa meal 1.5, calcium carbonate 0.5, sodium chloride 0.5, yeast powder 2, and cod liver oil 2 parts.

The presence of the compound in the precipitate which formed on acidification of the urine was demonstrated by the following experiment. The urine was made acid to Congo red by the addition of HCl, allowed to stand for 1 hour, and then centrifuged. The precipitate was drained, dried on a pad of filter paper, and then refluxed with chloroform for 6 hours. The chloroform extract was filtered and concentrated to a small volume by distillation. The remaining chloroform was removed by evaporation under reduced pressure. The residue was purified by crystallization from ethanol, from glacial acetic acid, and from cyclohexane. The crystalline product thus obtained was a pale brown color and melted at 268°. By this method 0.098 gm. of the compound was obtained from the precipitate which formed on acidification of the urine of six rats which had ingested 4.0 gm. of acenaphthene as a result of consuming a diet containing 1 per cent of the hydrocarbon.

*Properties*—The isolated compound, m.p. 269°, appeared to be insoluble at room temperature in water and dilute HCl. It was soluble in dilute NaOH solution and the solution when acidified gave a flocculent crystalline precipitate. It was soluble in ether, acetone, benzene, and chloroform. No residue was obtained on ignition of the compound. Nitrogen, sulfur, and halogens were found not to be present in the compound and Ehrlich's diazo test for phenolic groups yielded a negative result.

*Identification*—The melting point<sup>4</sup> and properties of the compound suggested that it was naphthalic anhydride,  $C_{12}H_6O_3$ . Naphthalic anhydride dissolves in concentrated sulfuric acid to give a yellow solution with a blue fluorescence (4) and this property was shown by the isolated compound. The results of elementary analyses of a sample of the compound suggested that it contained small amounts of naphthalic acid. The compound was, therefore, heated at 110° and when analyzed it yielded the following results.

$C_{12}H_6O_3$ . Calculated, C 72.73, H 3.05; found, C 72.69, H 3.14

The identity of the isolated compound as naphthalic anhydride was established by the synthesis of this compound and by the preparation of derivatives of the isolated and synthetic compounds with phenylhydrazine and with *o*-phenylenediamine.

Naphthalic acid was synthesized by the method of Graebe and Gfeller (6) and converted to the anhydride.

$C_{12}H_6O_3$ . Calculated, C 72.73, H 3.05; found, C 72.53, H 3.27

The synthetic naphthalic anhydride melted at 269° and when mixed with the compound obtained from urine the melting point was not depressed.

The preparation of N-anilinonaphthalimide, m.p. 218.5°, has been de-

<sup>4</sup> The melting points recorded for naphthalic anhydride in the literature vary widely; e.g., 266° (4), 269° (5), 274° (corrected) (6).



scribed by Jaubert (7). This compound was prepared by a different method from that used by Jaubert. A solution of 25 mg. of synthetic naphthalic anhydride and 14 mg. of phenylhydrazine in 6 ml. of benzene was refluxed for 45 minutes, the solvent was removed by evaporation, the residue was extracted with hot ethanol, and the material which separated from the ethanol on cooling was crystallized from benzene. A yield of 16 mg. of N-anilidonaphthalimide, m.p. 215–216°, was obtained.

$C_{18}H_{12}O_2N_2$	Calculated.	C 74.99, H 4.20, N 9.72
	Found.	" 75.32, " 4.42, " 9.80

The application of this procedure to the compound obtained from urine yielded 14 mg. of product, m.p. 215–216°. The melting point was not depressed when the compound was mixed with N-anilidonaphthalimide.

$C_{18}H_{12}O_2N_2$	Calculated	C 74.99, H 4.20, N 9.72
	Found	" 74.99, " 4.54, " 9.68

1',8'-Naphthoylene-1,2-benzimidazole, m.p. 204–205°, was prepared according to the method of Rule and Thompson (8) by the interaction of synthetic naphthalic anhydride and *o*-phenylenediamine in boiling acetic acid solution.

$C_{18}H_{10}ON_2$	Calculated	C 79.98, H 3.73, N 10.36
	Found.	" 79.90, " 3.84, " 10.25

Under the same conditions 20 mg. of product were obtained from 25 mg. of the compound isolated from urine.

$C_{18}H_{10}ON_2$	Calculated	C 79.98, H 3.73, N 10.36
	Found.	" 79.79, " 3.84, " 10.15

The compound melted at 204–205° and when mixed with 1',8'-naphthoylene-1,2-benzimidazole the melting point was not depressed.

On the basis of the various findings described above it is concluded that the compound, m.p. 269°, isolated from the urine of rats dosed with acenaphthene was the anhydride of naphthalene-1,8-dicarboxylic acid.

#### DISCUSSION

Conversion of naphthalic acid to its anhydride takes place readily (4) and the isolation procedures applied to the urine in the present work were such that they would yield naphthalic anhydride even though the acidified urine contained naphthalic acid. Although the findings can be explained on the ground that the urine when excreted contained naphthalic acid or its salts, the evidence at present available does not exclude the possibility that it contained conjugated compounds which were decomposed by acid

to yield naphthalic acid or naphthalic anhydride. Whatever the nature of the compound or compounds excreted in the urine, the fact that naphthalic anhydride was obtained from the urine under the conditions used in the present work suggests that the 5-membered carbon ring in acenaphthene undergoes fission in the rat. In view of the fact that the acenaphthene structure forms an integral part of the cholanthrene molecule, there arises the question of whether the 5-membered ring of cholanthrene, or of methylcholanthrene, undergoes fission in the animal body.

The authors are indebted to Mr. Michael Edson who performed the microanalyses reported above.

#### SUMMARY

The anhydride of naphthalene-1,8-dicarboxylic acid has been obtained from the urine of rats dosed with acenaphthene. Its identity has been established by synthesis and by the preparation of N-anilinonaphthalimide and 1',8'-naphthoylene-1,2-benzimidazole.

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# THE FECAL EXCRETION OF POLYCYCLIC HYDROCARBONS FOLLOWING THEIR ADMINISTRATION TO THE RAT

By L. H. CHANG\*

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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In biochemical studies of certain carcinogenic and non-carcinogenic polycyclic hydrocarbons various workers (*e.g.* (1-5)) have administered these compounds to animals by stomach tube or by admixture in the diet. The fact that various hydrocarbon derivatives have been isolated from the urine of animals dosed in this way has established that the absorption of polycyclic hydrocarbons or derivatives formed from them in the alimentary tract can take place. Little information has been available hitherto, however, concerning the fecal excretion of polycyclic hydrocarbons following their administration under the above conditions. In order to gain further information on this subject the present study of the fecal excretion of various hydrocarbons by the rat was undertaken. The compounds studied were the non-carcinogenic hydrocarbons, naphthalene, acenaphthene, phenanthrene, anthracene, and chrysene, and the carcinogenic hydrocarbons, 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, and methylcholanthrene. Each hydrocarbon was administered to rats by two methods; namely, by inclusion in the diet, and by administration in the form of a fine suspension by stomach tube. The results obtained reveal that when administered by these methods the above polycyclic hydrocarbons differ widely with respect to the proportions of the doses which are excreted in the feces. For example, whereas no fecal excretion of naphthalene was found to occur after the administration of this compound, almost all of a dose of 1,2,5,6-dibenzanthracene was found to be excreted in the feces.

## *Methods*

In order to determine the amount of a given polycyclic hydrocarbon in rat feces a simple gravimetric method was developed. The main steps in the method were as follows: The feces were extracted with ether, the ether extract was washed with acid and with water, evaporated, and the residue so obtained was saponified with alcoholic potassium hydroxide. The hydrocarbon was isolated from the saponified mixture and weighed.

The feces which were to be analyzed for their content of a hydrocarbon were sifted to free them from fallen fur and were then ground in a mortar.

\* Sino-British Fund Fellow. Present address, Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut.

A sample of 2 to 3 gm. of ground feces was weighed into an extraction thimble and continuously extracted with 150 ml. of ether in a Soxhlet apparatus for 6 hours. This period of extraction was found to be adequate for the complete extraction of the hydrocarbons studied. The ether extract was transferred to a 250 ml. separatory funnel, and washed with two 50 ml. portions of 2 *N* HCl and then with two 50 ml. portions of water. The ether layer was separated, transferred to a flask, and carefully distilled on a water bath. The residue thus obtained was saponified by refluxing with 5 ml. of alcoholic potassium hydroxide (40 gm. of KOH in 1 liter of 95 per cent ethanol) on a steam bath for 1 hour, during which time the flask was frequently agitated in order to facilitate reaction. The saponified mixture was then treated by a procedure depending upon which hydrocarbon was being determined.

In the case of anthracene, chrysene, 3,4-benzpyrene, methylcholanthrene, or 1,2,5,6-dibenzanthracene, all of which are almost completely insoluble in dilute ethanol, the hydrocarbon was precipitated by diluting the saponified mixture with water. The hot saponified mixture, which frequently contained crystals of hydrocarbon, was diluted with water until the liquid became slightly cloudy. It was allowed to cool with occasional shaking and was then diluted to a volume of 50 ml. with water. The precipitated hydrocarbon was collected on a sintered glass filter and washed with hot water to remove adhering traces of soaps. After air had been drawn through the filter for a short time, the precipitate was dissolved in ether. The ether solution was dried over anhydrous calcium chloride and filtered. The filtrate and ether washings were collected in a weighed flask, and the ether was removed by careful distillation on a water bath. The flask and its contents were then dried in a desiccator and weighed.

In the case of naphthalene, acenaphthene, or phenanthrene, all of which are appreciably soluble in dilute ethanol, the dilution of the saponified mixture with water did not bring about complete precipitation of the hydrocarbon. In the determination of these hydrocarbons the saponified mixture was diluted with water as described above and the filtrate and washings obtained after the filtration of the precipitated hydrocarbon were combined and steam-distilled in order to recover dissolved hydrocarbon. In order to prevent foaming of the liquid during distillation 10 ml. of 20 per cent calcium chloride solution were added to precipitate soaps. The distillation was continued until the distillate as it condensed was no longer turbid. The distillate was extracted twice with 50 ml. portions of ether. The combined ether extracts were washed twice with water and added to the ether solution of the hydrocarbon precipitated by dilution of the saponified mixture. The combined ether solutions were dried, evaporated, and the residue weighed as described above.

In no experiment in which the method (with or without steam distillation) was applied to a series of samples of normal rat feces, each of which weighed 5 gm., was the final residue found to weigh more than 1 mg.

The method was also applied to the analysis of samples of normal rat feces to each of which a known amount of hydrocarbon had been added. In order to determine the influence of a non-saponifiable compound on the results obtained, some experiments were performed in which cholesterol was added to the feces in addition to hydrocarbon. The addition of cholesterol to the feces was found to have no significant effect on the results

TABLE I  
*Recovery of Polycyclic Hydrocarbons Added to Rat Feces  
(with or without Added Cholesterol)*

Hydrocarbon added	Weight of feces	Weight of cholesterol added	Weight of hydrocarbon added	Weight of final residue	Final residue expressed as per cent of hydrocarbon added
	gm	mg	mg	mg	
Naphthalene	3 0		30.4	28.6	94
Acenaphthene	3.0		23 1	21 1	92
Anthracene	2.0		59 5	58.0	97
“	3.0		19 0	19.3	102
“	5.0		10 0	10.1	101
“	3.0	20 6	27 9	28 5	102
Phenanthrene	2 0		5 8	6 0	103
“	2.0		12 4	11.9	96
“	5 0		24 7	25 1	102
Chrysene	3 0		21 0	20 5	98
“	3 0		36 1	36 0	100
“	3.0	17 0	21 0	21 9	104
1,2,5,6-Dibenzanthracene	3.0		27 4	27 2	99
“	3 0		20 8	21.1	101
3,4-Benzpyrene	3.0		29 5	30.6	104
“	5 0		42.5	40.7	96
Methylcholanthrene	3 0		36 0	35.9	100
“	3 0	26.3	17.6	17 6	100

obtained. The results obtained are summarized in Table I. They show that with the exception of naphthalene and acenaphthene, both of which are somewhat volatile, the maximum errors in the recovery of the added hydrocarbons did not exceed  $\pm 4$  per cent.

### Results

Two series of experiments were performed in which polycyclic hydrocarbons were administered singly to rats. In the first series of experiments the hydrocarbons were included in the diets of the animals and in the second they were administered by stomach tube.

*Feeding Experiments*—The procedure in each experiment was as follows: The hydrocarbon was incorporated in the stock colony diet<sup>1</sup> to the extent of 0.2 or 1 per cent by weight and fed to four male white rats each weighing about 300 gm. No rat received more than one hydrocarbon. The rats were housed in pairs in metabolism cages which permitted the separate collection of urine and feces. The feces were inaccessible to the animals. The rats were fed in a separate cage for a 1 hour period twice daily at

TABLE II  
*Fecal Excretion of Polycyclic Hydrocarbons by Rat Following Their Ingestion in Diet*

Hydrocarbon added to diet	Amount of hydrocarbon in diet	Intake of hydrocarbon		Hydrocarbon in feces	
	per cent	mg.	mg	per cent of intake	
Naphthalene	1.0	535	0	0	
"	1.0	770	0	0	
Acenaphthene	1.0	715	43	6	
Anthracene	1.0	830	694	84	
"	1.0	605	498	82	
"	0.2	270	144	53	
Phenanthrene	1.0	720	46	6	
"	1.0	635	25	4	
Chrysene	1.0	850	686	81	
"	1.0	920	665	72	
"	1.0	440	376	85	
"	0.2	320	204	64	
1,2,5,6-Dibenzanthracene	1.0	780	710	91	
"	1.0	815	732	90	
"	1.0	800	715	89	
"	0.2	365	297	81	
3,4-Benzpyrene	1.0	525	232	44	
"	1.0	400	174	44	
"	1.0	480	184	38	
Methylcholanthrene	1.0	820	574	70	
"	1.0	660	474	72	
"	1.0	740	426	58	

10 a.m. and 5 p.m. The food intake at each feeding period was determined and the diet containing the hydrocarbon was fed to the rats until they had ingested at least 250 mg. of the hydrocarbon. The rats were then placed on the stock diet for 2 days and the collection of feces was continued during this period. Feces collected subsequently did not contain significant

<sup>1</sup> The composition of the stock diet was as follows: ground corn 50, dried milk 25, linseed meal 15, casein 3.5, alfalfa meal 1.5, calcium carbonate 0.5, sodium chloride 0.5, yeast powder 2, and cod liver oil 2 parts.

amounts of hydrocarbon. The feces collected during the whole experiment were mixed, sifted, ground, weighed, and duplicate samples were analyzed for their hydrocarbon content. The results obtained are summarized in Table II.

In every experiment in which the diet contained 0.2 per cent of hydrocarbon it was found that the percentage of ingested hydrocarbon which was excreted in the feces was lower than that excreted on the diet which contained the same hydrocarbon to the extent of 1 per cent. In most experiments the inclusion of hydrocarbon in the diet led to a fall in food intake, which varied according to the nature of the hydrocarbon in the diet. In consequence of this the level of hydrocarbon intake differed in different

TABLE III

*Fecal Excretion of Polycyclic Hydrocarbons by Rat Following Their Administration by Stomach Tube*

Hydrocarbon (200 mg) administered	Hydrocarbon in feces	
	mg.	per cent of intake
Naphthalene ...	0	0
Acenaphthene	12	6
"	25	13
Anthracene	123	64
"	147	74
Phenanthrene	11	6
"	13	7
Chrysene	174	87
"	163	82
1,2,5,6-Dibenzanthracene	200	100
"	185	93
3,4-Benzpyrene	116	58
"	110	55
Methylcholanthrene	135	68
"	138	69

experiments, a fact which complicates the comparison of the results obtained. For this reason the dosing experiments described below were undertaken.

*Dosing Experiments*—In order to compare the fecal excretion of hydrocarbons at the same level of intake, experiments were performed in which two male white rats, each weighing about 300 gm., each received a single dose of 0.1 gm. of hydrocarbon by direct administration. No rat was dosed with more than one hydrocarbon. The procedure in each experiment was as follows: A weighed quantity of hydrocarbon was ground in a mortar with sufficient 1 per cent starch solution to give a thin paste. This was then diluted with water to give a suspension which contained 0.1 gm.



of hydrocarbon per ml. 1 ml. of this suspension was then given by stomach tube to each rat. The two rats were kept together in a metabolism cage in which they received food and water *ad libitum*. In order to minimize the scattering of food the animals were fed on a stiff dough made up of equal parts of the stock colony diet and 5 per cent starch solution. The feces excreted during the 3 days after the administration of the hydrocarbon were collected and analyzed. The feces collected after this period were found not to contain measurable amounts of hydrocarbon. The results obtained are summarized in Table III.

#### DISCUSSION

Under the conditions described above the various polycyclic hydrocarbons studied showed marked differences in the extents to which they were excreted in the feces of the rat. Following the administration of naphthalene, the simplest hydrocarbon studied, no unchanged hydrocarbon was detected in the feces. Unchanged hydrocarbon was recovered from the feces in every experiment, however, in which more complex hydrocarbons were administered. Following the ingestion by rats of diets containing 1 per cent of a hydrocarbon the mean percentage recoveries of the hydrocarbons from the feces were as follows: phenanthrene 5, acenaphthene 6, 3,4-benzpyrene 42, methylcholanthrene 67, chrysene 79, anthracene 83, 1,2,5,6-dibenzanthracene 90. In experiments in which rats each received a suspension of 0.1 gm. of a hydrocarbon in starch solution by stomach tube the mean percentage recoveries of the hydrocarbons from the feces were as follows: phenanthrene 7, acenaphthene 10, 3,4-benzpyrene 57, methylcholanthrene 69, anthracene 69, chrysene 85, 1,2,5,6-dibenzanthracene 97. Although the results obtained with different methods of administering the hydrocarbons resemble one another fairly closely, those obtained from the dosing experiments serve as a better basis for comparing the fecal excretion of different hydrocarbons than do those obtained from feeding experiments, owing to differences in the level of hydrocarbon intake in the latter experiments.

In the experiments of Boyland and his coworkers with anthracene (3) and with 1,2,5,6-dibenzanthracene (4) these compounds were administered orally to rats and hydrocarbon derivatives were isolated from the urine of the dosed animals. In neither case did the quantities of the isolated compounds represent more than a small proportion of the hydrocarbon administered and the fate of a major portion of the dose of hydrocarbon therefore remained in doubt. The results of the present investigation show that a large proportion of a dose of anthracene or 1,2,5,6-dibenzanthracene administered orally to the rat is excreted in the feces.

## SUMMARY

Suitable analytical methods have been developed and a quantitative study has been made of the fecal excretion of polycyclic hydrocarbons by the rat following their administration by admixture in the diet or by stomach tube.

The hydrocarbons studied were naphthalene, acenaphthene, anthracene, phenanthrene, chrysene, 3,4-benzpyrene, methylcholanthrene, and 1,2,5,6-dibenzanthracene.

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## FURTHER EVIDENCE FOR THE EXISTENCE OF SPECIFIC DIETARY ESSENTIALS FOR THE GUINEA PIG\*

BY G. J. MANNERING, M. D. CANNON, H. V. BARKI, C. A. ELVEHJEM, AND  
E. B. HART

*(From the Department of Biochemistry, College of Agriculture, University of Wisconsin,  
Madison)*

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In a previous report from this laboratory (1) it was shown that young guinea pigs would not survive or grow when fed the purified diets commonly employed successfully in the nutrition of the rat. At this time three new dietary essentials required by the guinea pig were indicated; namely, a factor found in grass, a second factor existing in yeast, and a third in milk. Woolley (2) has demonstrated that, in addition to the water-soluble vitamins now recognized in pure form, the guinea pig requires at least three other dietary essentials. These factors were designated as GPF-1, present in a 50 per cent ethanol extract of linseed oil meal, GPF-2, a factor remaining in the alcohol-extracted linseed oil meal, and GPF-3, a third factor postulated on the basis that the addition of linseed oil meal alone to a purified ration would not support adequate growth or survival. Dried grass only partially satisfied the GPF-3 requirement.

Although the existence of the factors in yeast, grass, and milk could be repeatedly demonstrated, the large numbers of animals required rendered the procedure impractical for the numerous assays necessary in the fractionation of the individual factors. This technical difficulty was due largely to the presence in any one of the above materials of considerable amounts of one or both of the other two dietary essentials. A more practicable assay procedure was sought in the methods employed by Woolley.

That linseed oil meal contributes to the nutrition of the guinea pig has been definitely confirmed, but the clear differentiation of two factors by ethanol extraction has been only partially successful. However, data will be given showing that, in addition to the factor or factors present in linseed oil meal, another dietary essential is required by the guinea pig. The distribution and some of the properties of this factor will be discussed.

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## EXPERIMENTAL

*General Procedure*

When young guinea pigs from our stock colony weighed between 150 and 200 gm. (8 to 16 days of age), they were placed in metal cages with  $\frac{1}{2}$  inch mesh screen bottoms and maintained on experiment for a period of 6 weeks. The animals were weighed three times weekly and fed and watered twice daily. They were distributed equitably as regards weight and sex among the groups.

The basal ration used, No. S-29, was similar to Ration S-28, previously shown to be inadequate for the nutrition of the guinea pig (1). It consisted of the following: sucrose 72 gm., ethanol-extracted reprecipitated casein 20 gm., salts (3) 4 gm., corn oil 4 gm., thiamine 0.25 mg., riboflavin 0.35 mg., pyridoxine 0.25 mg., pantothenic acid 1.0 mg., nicotinic acid 5.0 mg., and choline 100 mg. When the basal ration was supplemented with appreciable amounts of dry material, the sucrose content was correspondingly decreased. The rations were mixed frequently and stored in the refrigerator. All animals received 20 mg. of ascorbic acid orally every other day and 1 mg. of  $\alpha$ -tocopherol and 0.05 cc. of haliver oil per week. The  $\alpha$ -tocopherol and haliver oil were diluted and mixed with corn oil just previous to feeding.

*Evidence for New Factors Required by Guinea Pig (Table I)*—Guinea pigs fed the basal ration failed to grow and died. The average survival time was 27 days. When 25 per cent of linseed oil meal was added (Ration S-44), the animals grew at a suboptimal rate for the first 2 or 3 weeks of the experiment, then rapidly lost weight, and died, with an average survival time of 34 days. Although the appearance of the guinea pigs was improved and their survival somewhat prolonged by the feeding of linseed oil meal, Ration S-44 was still lacking in some essential factor. The animals became very emaciated before dying but no specific symptoms or lesions were noted. Growth and survival were not improved when the linseed oil meal ration was modified by doubling the content of the crystalline B vitamins, adding 0.5 per cent of cystine and 10 per cent of gelatin, or by increasing the level of casein to 30 per cent of the diet. Likewise, the daily addition of 25 mg. of inositol, 0.4 mg. of 2-methyl-1,4-naphthoquinone, and 3  $\gamma$  of biotin (biotin liver concentrate) had no beneficial effect in alleviating the deficiency.

This substantiates the work of Woolley who was unable to obtain sustained growth in guinea pigs on a diet similar to Ration S-44. In his experiments guinea pigs fed this ration grew normally for 3 or 4 weeks, suddenly ceased growing, and died within a week or two. Dried grass reduced but did not eliminate this condition. He reports that no material

was found which would prevent the deficiency when fed in relatively small amounts.

We have fed various materials in an attempt to obtain a source of the factor apparently required by the guinea pig in addition to the two factors postulated as being present in adequate amounts in linseed oil meal. Dried grass (Cerophyl) fed at a level of 2 per cent proved to be an inadequate source of this factor, but when increased to a level of 8 per cent fair growth resulted. Grass juice powder<sup>1</sup> at a 4 per cent level proved inadequate but at 8 per cent excellent growth was obtained. Normal growth resulted when solubilized liver powder<sup>2</sup> was fed at 4 per cent. Increasing the level

TABLE I  
*Evidence for New Factors Required by Guinea Pig*

Ration No	No of animals	Survivors at 6 wks.	Average daily gain*
			gm
S-29†	28	1	
S-29 + 25% linseed oil meal (Ration S-44)	23	6	-0.6
S-29 + 4% solubilized liver extract	13	11	1.1
S-44 + 2% dried grass	4	2	0.7
S-44 + 8% " "	4	3	2.0
S-44 + 4% grass juice powder	7	2	1.8
S-44 + 8% " " "	8	8	4.3
S-44 + 4% solubilized liver extract	9	9	4.3
S-44 + 8% yeast‡	4	4	3.0
S-44 + 20 cc. milk per day.	15	12	1.5
S-44 + 10% skim milk powder	3	2	3.8

\* Calculated for the 6 weeks experimental period, including only those animals surviving 6 weeks.

† Basal Ration S-29 described in the text

‡ Northwestern Yeast Foam.

of liver powder to 8 per cent did not result in an additional growth response. Yeast, milk, and skim milk powder are fair sources of this factor.

The poor growth resulting when solubilized liver was fed in the absence of linseed oil meal is good evidence that solubilized liver alone at a 4 per cent level does not supply all the factors required by the guinea pig. We have been unable to demonstrate with any consistency the separation of two factors in linseed oil meal by means of ethanol extraction, as reported

<sup>1</sup> Grass juice powder is the spray-dried pressed juice of cereal grasses. 1 gm. of grass juice powder is equivalent to about 4 gm. of dried grass (Cerophyl).

<sup>2</sup> The solubilized liver powder (Fraction L) is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water-soluble by enzyme action.

by Woolley. Differences in the commercial methods of processing linseed oil meal may account for this discrepancy. Some question may arise, then, as to whether or not the factor found in solubilized liver powder and certain other materials and not in sufficient quantity in linseed oil meal is actually a *third* factor. However, for convenience, this factor will be referred to as GPF-3.

*Properties of GPF-3*—Studies on some of the properties of the third factor have been made and the resulting data are presented in Table II.

TABLE II  
*Properties of GPF-3*

The ration employed was the purified Ration S-29 described in the text plus 25 per cent linseed oil meal.

Supplement	No of animals	Survivors at 6 wks.	Average daily gain
			gms
Acid-autoclaved S.L.P., * 4%	6	0	
Alkaline autoclaved S.L.P., 4%	6	6	5.5
Aerated S.L.P., 4%	3	3	3.6
Acid ether extract of S.L.P. $\approx$ 12%	3	0	
Alkaline ether extract of S.L.P. $\approx$ 12%	3	2	1.2
Norit eluate of G.J.P.† $\approx$ 8%	7	4	2.8
“ filtrate of G.J.P. $\approx$ 8%	7	7	3.6
“ eluate of S.L.P. $\approx$ 12%	3	3	2.5
Filtrate from Pb ppt. of S.L.P. $\approx$ 12%	3	3	4.2
Residue “ “ “ “ $\approx$ 12%	3	3	2.7
Filtrate “ “ “ “ G.J.P. $\approx$ 12%	3	3	5.4
Residue “ “ “ “ $\approx$ 12%	3	1	3.6
Fullers' earth filtrate of Pb filtrate of S.L.P. $\approx$ 12%	3	3	3.8
“ “ adsorbate of Pb filtrate of S.L.P. $\approx$ 12%	3	0	
Super Filtrol filtrate of G.J.P. $\approx$ 8%	3	3	4.4
“ “ adsorbate of G.J.P. $\approx$ 8%	3	3	1.9

\* Solubilized liver powder.

† Grass juice powder.

Autoclaving under acid conditions completely destroyed the factor as far as could be determined by our assay procedure. This treatment was accomplished by dissolving the solubilized liver powder in water, adjusting to pH 1.0 with  $H_2SO_4$ , and autoclaving for 20 minutes at 15 pounds pressure. Autoclaving under similar conditions at a pH of 11.0 (NaOH) had no recognizable destructive effect on the GPF-3 activity of liver powder. Aeration, by passing air for 36 hours at room temperature through a solution of liver powder, had little effect on the activity. The factor could not be extracted with ether from either acid or alkaline solution.

The above properties suggested the possible identity of GPF-3 and folic acid. Crude folic acid preparations were therefore prepared from both grass juice powder and solubilized liver powder according to the method of Hutchings *et al.* (4), which involves adsorption on norit at pH 3.0 and elution with an ammonia-ethanol mixture. The norit eluates, containing 50 to 60 per cent of the original folic acid, when fed at high levels resulted in suboptimal growth. The filtrate of grass juice powder remaining after norit treatment, though possessing only about 5 per cent of the original folic acid activity, was superior to the norit eluate fraction as a source of GPF-3. The folic acid content of these fractions was assayed by employing essentially the method of Mitchell and Snell (5), which depends upon the growth response of *Streptococcus lactis* R.<sup>3</sup> The growth-stimulating property of the fractions for *Lactobacillus casei* closely paralleled that for *Streptococcus lactis* R. That GPF-3 and folic acid are not identical seems highly probable. A further argument in favor of this view is that the grass juice powder used in these experiments was about twice as active as a source of folic acid as was liver powder but was somewhat less active as a source of GPF-3.

Treatment with lead acetate at pH 4.5 has not yielded clear cut separations. Most of the factor remains unprecipitated, although appreciable amounts may be recovered from the lead precipitate by treating with hydrogen sulfide. The more active lead filtrates contained only 5 to 20 per cent of the original folic acid.

The factor could not be readily removed from the filtrate of the lead precipitate by means of adsorption on fullers' earth at pH 2.0. GPF-3 was poorly adsorbed on norit (pH 3.0) and on Super Filtrol (pH 2.0). The fullers' earth and Super Filtrol adsorbates were fed as such without attempts at elution.

#### DISCUSSION

The data presented above show that the guinea pig requires, in addition to the factors present in linseed oil meal, a third factor found in solubilized liver powder, grass juice powder, and other materials. This factor is assumed to be identical, at least by definition, with the GPF-3 described by Woolley (2). Since levels of linseed oil meal higher than 25 per cent were not fed, the question exists as to whether the proposed third factor is not in reality merely relieving a relative deficiency of GPF-1 or GPF-2, if either of these factors is not present in optimal amounts in linseed oil meal. This question has not been answered. Further doubt as to the identity of the above factor and GPF-3 as described by Woolley

<sup>3</sup> The folic acid assays were kindly performed by T. D. Luckey.



arises from his report that no source of GPF-3 could be found, while our data show a fairly wide distribution of this factor.

Non-parallel concentration of folic acid and the third factor strongly suggests that the two are separate entities. It is not known whether GPF-3 is identical with either of the chick factors B<sub>10</sub> or B<sub>11</sub> (6), although the latter seem more readily adsorbable on norit.

Hogan and Hamilton (7) have reported that the addition of 16 per cent of yeast to a purified ration satisfied all the growth requirements of the guinea pig. We have been unable to substantiate this finding (1). However, the diets they employed differed from those we have used in that they contained dextrin rather than sucrose as a source of carbohydrate and included a relatively high level of cellulose. Preliminary experiments indicate that although yeast alone does not supply all the essential factors required by the guinea pig the substitution of dextrin for sucrose and the inclusion of cellulose in the yeast ration prolong the survival of animals and allow fair, but suboptimal growth. This can be explained on the hypothesis that dextrin and cellulose promote the synthesis of essential factors by intestinal bacteria.

Owing to the previously mentioned technical difficulty in separating the two linseed oil meal factors, we have been unable to determine which of GPF-1, GPF-2, and GPF-3 are identical with the grass, yeast, and milk factors originally proposed by this laboratory (1). Yeast is a fairly good source of GPF-3. However, it is lacking in one or both of the linseed oil meal factors, since yeast alone when fed at a relatively high level will not support adequate growth. GPF-3 is apparently not the "grass juice factor" of Kohler *et al.* (8). The milk assayed in these experiments was the same type of winter milk that has been used as the basal diet in the assay of the "grass juice factor." Although milk allowed only fair growth when fed as a supplement (20 cc. per day) to the GPF-3-deficient ration, it is expected that a sufficient amount of the third factor would be provided when winter milk constituted the main bulk of the diet, as is the case in the original assay procedure for "grass juice factor." We have unpublished data showing that linseed oil meal will support excellent growth in the guinea pig when fed as a supplement to winter milk, indicating that one or both of the linseed oil meal factors is the "grass juice factor." Since both dried grass and linseed oil meal are good sources of the "grass juice factor" and dried grass is limiting in the third factor, the dried grass must be a better source of at least one of the linseed oil meal factors than it is of GPF-3. In the preparation of grass juice powder GPF-3 is concentrated sufficiently to make it a good source of the third factor.

## SUMMARY

In addition to the factors present in linseed oil meal a factor found in solubilized liver powder and grass juice powder is required by the guinea pig. The distribution and properties of this factor are discussed.

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# THE VALUE OF BIOTIN, FOLIC ACID CONCENTRATES, AND LIVER EXTRACT IN THE DIET OF RATS FED SUCCINYLSULFATHIAZOLE\*

By BEVERLY RANSONE† AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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It is now definitely established that the feeding of poorly absorbed sulfonamides such as sulfaguanidine (sulfanilylguanidine) and sulfasuxidine (succinylsulfathiazole) to rats receiving a synthetic ration decreases the rate of growth (1-15). Black, McKibbin, and Elvehjem (1) first showed that *p*-aminobenzoic acid counteracted the effect of sulfaguanidine and that liver extract allowed better growth than that obtained on the synthetic diet without the drug. Both sulfaguanidine and sulfasuxidine were found to induce a hypoprothrombinemia which could be prevented with liver extract, and the hypoprothrombinemia of sulfaguanidine was shown to be corrected with *p*-aminobenzoic acid or a folic acid concentrate (10). Martin (4) has reported the production of achromotrichia by feeding sulfaguanidine and the maintenance of color in sulfaguanidine-fed rats when a folic acid concentrate was added, and Nielsen and Elvehjem (5) showed that rats fed sulfasuxidine required a dietary source of biotin in addition to a folic acid concentrate. Pathological changes including leucopenia have been demonstrated in rats fed these drugs (7, 11, 12).

The inability of *p*-aminobenzoic acid to counteract sulfasuxidine was first shown by Welch (9) and has been further studied by Neumann, Krider, and Day (13), who showed that *p*-aminobenzoic acid does not prevent the biotin deficiency caused by sulfasuxidine. Totter and Day (14) have reported that xanthopterin counteracts the decreased growth and leucopenia obtained with sulfasuxidine. Wright and Welch (16) have reported a low level of pantothenic acid in the livers of rats fed sulfasuxidine, and that both a folic acid concentrate and biotin are required to raise the level to normal.

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† Eli Lilly and Company Fellow.

We wish to report at this time additional work performed in our laboratory during the past year on the action of biotin, folic acid concentrates, and liver extract in counteracting the effects of sulfasuxidine in the ration of the rat.

#### EXPERIMENTAL

Male rats of the Sprague-Dawley strain, 20 or 21 days old, weighing 35 to 45 gm. were fed the following ration: sucrose 76, casein 18, Salts 4 (17) 4, and corn oil 2. The following vitamins were added per 100 gm. of ration: thiamine 0.2 mg., pyridoxine 0.25 mg., riboflavin 0.3 mg., calcium pantothenate 2 mg., nicotinic acid 0.25 mg., and choline hydrochloride 0.1 gm. Each rat was given 2 drops of a 1:6 dilution of haliver oil in corn oil once a week. When sulfasuxidine was used, 0.5 gm. was added to 100 gm. of ration.

The folic acid concentrate used was prepared from liver Fraction L according to the method of Hutchings *et al.* (18), but the final norit adsorption was omitted. Both the concentrate and liver extract<sup>1</sup> were assayed for folic acid by the method of Mitchell and Snell (19); *Streptococcus lactis* was the test organism used, and solubilized liver<sup>2</sup> was the standard. The folic acid concentrate also showed considerable activity when it was assayed with *Lactobacillus casei*. The liquid supplements were pipetted daily into small dishes separate from food and water.

Each experimental group contained three rats, and all groups were kept on experiment for at least 5 weeks. The rats used for leucocyte counts were kept on experiment for longer periods of time.

In one series the rats were fed the sulfasuxidine ration described and 0.5  $\gamma$  of biotin per day as a concentrate.<sup>3</sup> One group received daily in addition a folic acid concentrate equivalent in folic acid content to 0.1 gm. of standard; a second group was given twice this quantity of folic acid concentrate. A third group received daily liver extract equivalent in folic acid content to 0.1 gm. of standard, and a fourth group was given twice this quantity of liver extract. The quantities of concentrate and liver extract were based upon *Streptococcus lactis* assay. The results of this series are summarized in Table I. Either the folic acid concentrate or the liver extract containing folic acid equivalent to 0.1 gm. of the standard when fed in conjunction with 0.5  $\gamma$  of biotin protected rats fed the sulfasuxidine ration. Rats given either of these supplements grew as well as or better than control rats receiving no drug and no supplement. The rats receiving liver extract and biotin grew better than rats fed the basal ration

<sup>1</sup> Wilson's, 1:20.

<sup>2</sup> Wilson's liver Fraction L.

<sup>3</sup> S. M. A., No. 1000.

without sulfasuxidine, and the rats given biotin and the lower level of folic acid concentrate grew just as well as the rats given biotin and the lower level of liver extract. When twice as much concentrate or twice as much liver extract was given, the rats fed the concentrate showed no additional growth response, but the rats given liver extract grew at a somewhat increased rate.

In a second series of rats, each of three groups received folic acid concentrate equivalent in folic acid content to 0.1, 0.05, and 0.025 gm. of the standard. Three additional groups received these same supplements but

TABLE I  
*Average Weekly Growth of Rats Fed Sulfasuxidine and Various Supplements*

	Equivalent in terms of standard	Series I	Series II	
		0.5 $\gamma$ biotin daily	0.5 $\gamma$ biotin daily	No biotin
	gm.	gm	gm.	gm
No sulfasuxidine		25		29
Sulfasuxidine, no supplement		12	13	12
Folic acid concentrate	0.2	27		
Liver extract	0.2	31		
Folic acid concentrate	0.1	28	25	20
Liver extract	0.1	27	31	28
Folic acid concentrate	0.05		22	20
Liver extract	0.05		27	22
Folic acid concentrate	0.025		20	14
Liver extract	0.025		22	17
Grass juice powder	0.1		26	22
Folic acid concentrate, acid-autoclaved	0.1			11
" " " autoclaved at neutrality	0.1			21
20 $\gamma$ xanthopterin			13	
40 " "			12	

were also given 0.5  $\gamma$  of biotin daily. Three other groups were given liver extract equivalent in folic acid content to 0.1, 0.05, and 0.025 gm. of standard. Each of three parallel groups received one of these same levels of liver extract and in addition 0.5  $\gamma$  of biotin. The results from this series are summarized in Table I. The rats which received biotin and either folic acid concentrate or liver extract containing as much folic acid as 0.1 gm. of standard grew well. Those receiving one-half this quantity of folic acid grew more slowly, and those given one-fourth the original amount grew at a definitely lower rate. In every group, whether the animals were fed adequate or inadequate levels of folic acid concentrate or liver extract, the growth was better when biotin was administered than when it was

omitted. The growth increase due to the biotin was in many groups 5 gm. per week at the end of 5 weeks. After 7 weeks on experiment about one-third of the rats which were given no biotin showed spectacle eye. After 9 to 10 weeks about one-third of them had lost hair around the mouth and on the throat and chest, and, in addition, showed a scaly brown dermatitis.

The ability of grass juice powder<sup>4</sup> to counteract sulfasuxidine was also determined. Two groups of rats received sulfasuxidine and grass juice powder equivalent in folic acid content to 0.1 gm. of standard. One of the groups was given 0.5  $\gamma$  of biotin, and the other received no biotin. The growth rate when grass juice powder and biotin were fed was similar to that obtained with liver extract and biotin or with a folic acid concentrate and biotin.

The folic acid concentrate was autoclaved at neutrality and in acid, and the autoclaved preparations were fed to rats to determine their ability to counteract the effects of sulfasuxidine. To prepare the acid-autoclaved concentrate, 1 part of concentrated HCl was added to 5 parts of folic acid concentrate and the resulting solution autoclaved at 15 pounds for 15 minutes. The HCl was removed under a vacuum, and the remaining solution was neutralized with  $\text{NH}_4\text{OH}$ . This preparation retained less than 2 per cent of its original folic acid activity as measured with *Streptococcus lactis*. The concentrate which was to be autoclaved at neutrality was neutralized to litmus and autoclaved at 15 pounds for 2 hours and 15 minutes. The folic acid activity of this preparation was nearly as great as that of the untreated concentrate. Each of these autoclaved concentrates was fed to a separate group of rats in quantities which before autoclaving were equivalent in folic acid content to 0.1 gm. of the standard. None of these animals was given biotin. The growth results are given in Table I. Acid autoclaving under the described conditions completely destroyed the ability of the concentrate to counteract the effects of the sulfasuxidine, and rats fed this supplement grew poorly. Autoclaving at neutrality, on the other hand, did not affect the ability of the concentrate to counteract the effects of sulfasuxidine.

Two groups of rats fed the sulfasuxidine ration and 0.5  $\gamma$  of biotin were fed respectively 20 and 40  $\gamma$  of xanthopterin daily. The growth rates of these rats were practically the same as those of the rats receiving sulfasuxidine without supplements. These data are given in Table I.

The effect of the autoclaved preparations as well as the effect of the untreated concentrate and liver extract upon the sulfasuxidine-induced leucopenia previously reported (7, 11, 12) was determined. Four groups of rats were fed the untreated folic acid concentrate, liver extract, acid-

<sup>4</sup> Cerophyl's spray-dried grass juice.

autoclaved concentrate, or concentrate autoclaved at neutrality. The folic acid concentrate and liver extract fed were equivalent in folic acid content to 0.1 gm. of standard, and the autoclaved preparations were fed in quantities which before autoclaving were equivalent in folic acid content to 0.1 gm. of standard. The leucocyte counts of these four groups of animals were determined. Typical values obtained are in Table II. The untreated folic acid concentrate or liver extract equivalent in folic acid content to 0.1 gm. of standard prevented the leucopenia. The concentrate autoclaved at neutrality was equally effective, but acid autoclaving completely destroyed the ability of the concentrate to prevent the leucopenia.

TABLE II

*Typical Leucocyte Counts of Rats Fed Sulfasuxidine*

Unless otherwise indicated all rats received sulfasuxidine. Supplements were equivalent in folic acid content to 0.1 gm. of standard, the assays being made on the material before autoclaving.

Supplement	Rat No	Days on experiment	Leucocytes per c mm.
No sulfasuxidine, no supplement	207	24	12,960
	207	59	12,960
	208	60	16,160
Sulfasuxidine, no supplement	211	25	6,320
	358	37	3,800
	267	54	1,070
Folic acid concentrate, not autoclaved	270	69	14,640
	269	69	9,240
Folic acid concentrate, acid-autoclaved	271	54	1,840
	272	46	2,700
Folic acid concentrate, autoclaved at neutrality	274	68	14,200
	275	68	11,120
Liver extract	181	63	11,240
	179	62	12,800

## DISCUSSION

It is well established that liver extract counteracts the effect of sulfasuxidine. Nielsen and Elvehjem (5) showed that the folic acid concentrate of Hutchings *et al.* (18) stimulated growth when fed to rats which had been receiving sulfasuxidine without supplement. Our experiments show that the concentrate protects during the entire period of rapid growth as well and approximates the effectiveness of the liver extract in this respect. The recent work of Welch and Wright (15) verifies this conclusion. In one series the rats fed sulfasuxidine, biotin, and the concentrate grew as well as those given sulfasuxidine, biotin, and liver extract. In a second series



the rats given liver grew 5 gm. per week more than those given the concentrate. In another series (data not given) the difference was 10 gm. There are several possible causes for these discrepancies. Both liver extract and the folic acid concentrate may contain substances other than the *Streptococcus lactis* factor which are active against sulfasuxidine effects. Both liver extract and the concentrate may contain more than one factor stimulating to *Streptococcus lactis*, and these factors may or may not be capable of counteracting the effects of sulfasuxidine. The recently published work of Pffner *et al.* (20) and Keresztesy *et al.* (21) suggests that more than one factor in the concentrates from liver and in liver may be active in counteracting the effects of sulfasuxidine. That at least one of the active compounds is active for *Streptococcus lactis* is suggested by the similar growth response obtained when the folic acid concentrate, liver extract, and grass juice powder are fed to separate groups in quantities containing as much folic acid as is present in 0.1 gm. of standard. Biotin fed to rats receiving different levels of folic acid concentrate or liver extract in every case showed a growth response.

The increased leucocyte counts obtained when rats were fed sulfasuxidine and either the folic acid concentrate or liver extract without added biotin show that the concentrate and liver extract are active in counteracting the sulfasuxidine-induced leucopenia even when biotin is not added to the ration. Here again the active ingredient is unknown, but the lack of response to the acid-autoclaved concentrate indicates that the counteracting principle is labile to heat in acid, and the normal white cell counts obtained when the concentrate which had been autoclaved at neutrality was fed indicate that the active substance is stable to prolonged heat at neutrality. Since growth rates were low when the white counts were low, and nearly equal to those of the controls when the white counts were normal, the substance or substances promoting growth may be identical with the factor or factors required to counteract the sulfasuxidine-induced leucopenia.

#### SUMMARY

A folic acid concentrate has been shown to approximate the activity of liver extract in counteracting the effects of sulfasuxidine when the concentrate and liver extract are fed at levels equal in *Streptococcus lactis* activity.

Biotin increased the growth rate when fed in conjunction with a folic acid concentrate or liver extract even when insufficient quantities of the concentrate or liver were fed.

The decreased growth and leucopenia induced in rats by feeding sulfasuxidine were counteracted by the untreated folic acid concentrate or by the same concentrate autoclaved at neutrality, but not by the concentrate when it had been autoclaved in acid.

**Xanthopterin fed in conjunction with biotin did not increase the growth rate of rats fed sulfasuxidine.**

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# ON THE HYDROLYSIS OF PROTEINS AND PEPTONES AT HIGH TEMPERATURES AND ON THE CATALYTIC EFFECT OF METAL IONS ON THE RATE OF HYDROLYSIS

By FRITZ LIEBEN\*

(From the Department of Surgery, Washington University School of Medicine, St. Louis)

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Since the time of the studies by Henriques and Gjaldbaek (1) and by Van Slyke (2) it has been known that a complete hydrolysis of casein, Witte's peptone, etc., (1.5 gm. in 50 cc.) can be performed in the autoclave at 150° with 3 N HCl in 1½ hours. These authors also observed that prolonged heating in the autoclave was followed by a decrease of the free amino nitrogen owing to the splitting off of amino groups as ammonia. In connection with their studies on racemization, Levene and Bass (3) attained a total hydrolysis of casein in sealed glass tubes at 125° with 5 N HCl after 4 hours (1 gm. in 10 cc.). More recently Borchers and Berg (4) reported on the hydrolysis of zein in the autoclave; they too observed the rise and subsequent fall of the free amino N. (Complete hydrolysis with 14, 25, and 33 per cent H<sub>2</sub>SO<sub>4</sub> required 30, 12, and 5 hours at 120°, 8, 6, and 3 hours at 140°, and 4, 2, and 1 hour at 160° respectively.)

In this paper, experiments are reported with the reflux condenser and the autoclave, which, in the latter case, have never been extended to more than 2 or at the most 3 hours; this is too short a time for the decomposition of significant amounts of amino groups. Sullivan and Hess (5) have further, in the course of their studies on the liberation of cystine in the form of cysteine from the protein complex, observed an acceleration of the hydrolysis rate in the presence of titanium trichloride (TiCl<sub>3</sub>).

## EXPERIMENTAL

The free amino N was determined in the volumetric Van Slyke apparatus as per cent of the total amino N after *complete* hydrolysis of 1 gm. of protein or peptone boiled in 100 cc. of 20 per cent H<sub>2</sub>SO<sub>4</sub> during 20 to 22 hours. 1 cc. of such a hydrolyzed solution gave as free amino N 0.93 mg. for casein, 0.77 mg. for gelatin, 0.92 mg. for Bacto-peptone, and 0.70 mg. for Witte's peptone. These figures (especially for gelatin) are lower than the figures given in the literature (3, 6), *i.e.* 1.0 to 1.10 mg. of NH<sub>2</sub>-N, but as they were obtained in a series of tests run in the same apparatus and under

\*Present address, Department of Chemistry, the Johns Hopkins University, Baltimore.

exactly the same conditions as all the following experiments, they were used as standard figures for calculation of the percental degree of hydrolysis.

TABLE I

*Correction of Amino N To Be Applied in Each Determination As a Function of Total Gas Volume Evolved during 6 Minutes*

Total gas evolved	Correction; free amino N
cc.	mg.
9	0.10
20	0.15
30	0.17
40	0.19

By drawing a curve and interpolating complete data can be obtained.

TABLE II

*Degree of Hydrolysis of Casein\**

Experiment No	Protein	H <sub>2</sub> SO <sub>4</sub>		Time	Temperature	Catalyst	Per cent of total hydrolysis			
		Volume	Concentration				Reflux condenser		Autoclave	
							With catalyst	Without catalyst	With catalyst	Without catalyst
	gm	cc	per cent	hrs	°C.					
1	2	100	20	1	160				100	
2	2	100	5	1	200				91	
3	2	100	5	1	160				77	
4	1	20†	15	4	B.p.	TiCl <sub>3</sub>	82	57		
5	1	20†	20	2½	"	"	77	62		
6	1	20†	20	4	"	"	91	75		
7	1	20†	20	5	"	"	97	89		
8	1	20†	20	5	"	SnCl <sub>2</sub>	98	85		
9	1	20†	20	2½	"	"	83	60		
10	1	20†	20	2½	"	SnCl <sub>4</sub>	80			
11	1	100	2	2	"	TiCl <sub>3</sub>	17	17		
12	1	100	2	2	200	"			84 68	
13	1	100	3	2	200	"			94 80	
14	1	100	5	2	200	"			98 88	
15	6	100	3	2	200	"			53 44	
16, 17	1	100*	0.1 N NaOH	2	200				31, 39	

\* The unheated original solution contained 5.5 per cent free amino N.

† The solution after being heated and previous to the determination was brought to 50 cc. (2 per cent casein solution) and filtered.

The free amino N, present in the protein or peptone solution *before* hydrolysis, was likewise measured.

TABLE III  
Degree of Hydrolysis of Gelatin

The unheated solution of 1 per cent gelatin in water contained 3.4 per cent free amino N.

Experiment No	Protein	H <sub>2</sub> O or acid	Acid concentration	Time	Temperature	Catalyst	Per cent of total hydrolysis			
							Reflux condenser		Autoclave	
							With catalyst	Without catalyst	With catalyst	Without catalyst
	gm.	cc.	per cent	hrs.	°C.					
1	2	100	H <sub>2</sub> O	1	200	TiCl <sub>3</sub>				20
2, 3	1	100	"	2	250					38, 32
4	1	100	"	2	200				65	32
5	1	100	"	3	200				90	
6	2	100	"	2	200				65	34
7	1	100	1 H <sub>2</sub> SO <sub>4</sub>	2	200				100	100
8	1	20*	H <sub>2</sub> O	2	B.p	"	22	9		
9	1	20*	5 H <sub>2</sub> SO <sub>4</sub>	2½	"	"	64	54		
10	2	100	H <sub>2</sub> O	2	"	"	15	7		
11	1	20*	2 H <sub>2</sub> SO <sub>4</sub>	2	"	"	37	24		
12	1	20*	2 "	2½	"	SnCl <sub>2</sub>	39			
13	1	20*	2 "	2½	"	SnCl <sub>4</sub>	45			

\* The solution after being heated and previous to the determination was brought to 50 cc. (2 per cent gelatin) and filtered.

TABLE IV  
Degree of Hydrolysis of Bacto-peptone and Witte's Peptone

The unheated solutions of Bacto-peptone, 1 per cent, and Witte's peptone, 1 per cent, in water contained 28 and 15 per cent free amino N respectively. 100 cc. of H<sub>2</sub>O were used in every case.

Experiment No	Peptone, 1 gm	Acid concentration	Time	Temperature	Catalyst	Per cent of total hydrolysis			
						Reflux condenser		Autoclave	
						With catalyst	Without catalyst	With catalyst	Without catalyst
		per cent	hrs.	°C.					
1	Bacto-peptone	1 H <sub>2</sub> SO <sub>4</sub>	2½	B.p.	TiCl <sub>3</sub>	37	28	-	
2	"		2	200	TiCl <sub>3</sub>				35
3	"		2	200				82	61
4	Witte's		2	B.p.	"	27	20		
5	"		2	200	"			51	30

In the determinations with the Van Slyke apparatus it was observed that the blank gas volume, the N equivalent of which must be subtracted from the value found in each determination, varies with the *total* volume of gas ( $\text{NO} + \text{N}_2$ ) evolved during the determination (6 minutes). These figures will vary too according to the apparatus used and are, for the following experiments, given in Table I.

The results for casein (Hammarsten, Merck), gelatin (Pfanstiehl), Bacto-peptone (Difco), and Witte's peptone (Rostock, pept. siccum) are given in Tables II to IV. After being heated on the reflux condenser or in the autoclave the solutions were filtered before the determinations were carried out. Pressures of 240 to 280 pounds per sq. in. were obtained with the autoclave by heating to  $200^\circ$ ; as catalysts 1 cc. of 20 per cent aqueous  $\text{TiCl}_3$  or 0.3 gm. of  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  or 0.5 gm. of  $\text{SnCl}_4 \cdot 5\text{H}_2\text{O}$  was used and found to be about equally effective. No catalytic effect could be demonstrated with Cu, Ni, or Mn salts or with metallic selenium in the reflux condenser experiments.

#### DISCUSSION

The experiments show that a complete or practically complete hydrolysis of casein can be achieved (a) by heating a 2 per cent solution in 20 per cent sulfuric acid in the autoclave for 1 hour (Table II, Experiment 1) at  $160^\circ$  as has also been reported by former investigators (1, 2), (b) by boiling a 5 per cent solution in 20 per cent  $\text{H}_2\text{SO}_4$  on the reflux condenser for 5 hours in presence of  $\text{TiCl}_3$  or  $\text{SnCl}_2$  (Experiments 7, 8), (c) by heating a 1 per cent solution with the above catalyst in 5 per cent  $\text{H}_2\text{SO}_4$  in the autoclave for 2 hours at  $200^\circ$  (Experiment 14). In 0.1 N alkali about one-third of the casein is hydrolyzed under the conditions indicated (Experiments 16, 17). A 100 per cent hydrolysis of gelatin in 1 per cent  $\text{H}_2\text{SO}_4$  is obtained in the autoclave at  $200^\circ$  in 2 hours (Table III, Experiment 7) even without a catalyst. A 1 or 2 per cent solution of gelatin in *water* with a catalyst is hydrolyzed in the autoclave at  $200^\circ$  to the extent of 65 per cent in 2 hours and of 90 per cent in 3 hours (Experiments 4 to 6). It is evident that a great number of other variations, such as temperature in the autoclave, amount of catalyst used, time of heating, and concentrations of substrate and acid would lead to similar results. However, the importance of *low* initial substrate concentration should be stressed. The autoclave experiments were all done in 1 or 2 per cent protein solution, with exception of Experiment 15 (Table II) in which an initial concentration of 6 per cent had the effect of lowering the degree of hydrolysis under pressure (with and without a catalyst) to nearly one-half of the amount given in the quite analogous Experiment 13 with a 1 per cent initial protein concentration. This essential rôle of the initial concentration applies of course equally to hydrolysis with the reflux condenser.

It was to be expected that the two peptones on account of their lower molecular weights would be rather more resistant to the hydrolytic agent; this was found to be the case if one considers the free amino N of 28 per cent and 15 per cent, respectively, present in the original unheated solution (7).

I want to thank Mr. M. Greenberg for technical assistance.

#### SUMMARY

Data are reported on the hydrolysis of casein, gelatin, Bacto-peptone, and Witte's peptone under different experimental conditions with the reflux condenser and in the autoclave.  $\text{TiCl}_3$ , as well as  $\text{SnCl}_2$  and  $\text{SnCl}_4$ , increases the rate of hydrolysis to an appreciable degree, while Cu, Ni, and Mn salts are without effect. It is noteworthy that relatively so short a time is required for 100 per cent hydrolysis of the protein in the presence of the catalysts, also that the 90 per cent cleavage of gelatin (1 per cent) in pure water may be effected in 3 hours. The peptones, as was to be expected, prove more resistant.

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# ON THE BIOLOGICAL SYNTHESIS OF PURINES AND PYRIMIDINES\*

BY FREDERICK W. BARNES, JR., AND RUDOLF SCHOENHEIMER†

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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That the biological formation of body purines does not require the presence of preformed purines in the diet has been shown for the chicken embryo (1, 2), the silkworm embryo (3), for a number of growing mammals (4-6), for the Dalmatian coach hound (7), for adult dogs (8), and for the adult human being (9). The identity of the precursors for purine synthesis has not been definitely established. Arginine and histidine (10-13), glycine, alanine, glutamic acid, aspartic acid, asparagine (14-17), ammonia (15-19), and proteins in general (20-22, 14, 23, 24, 15, 25, 13, 26) have all been suggested on the basis of metabolic balance experiments and tissue slice techniques. Investigations on the rôle of urea as a precursor have given contradictory results (19, 27-29). The biological synthesis of pyrimidine compounds and the possible rôle of pyrimidines in the formation of purines are subjects on which evidence has been difficult to obtain.

In an attempt to gain further insight into the manner in which these nitrogenous bases are formed in animals, we have fed to rats and pigeons compounds marked with an excess of the nitrogen isotope of mass 15. Pigeons were included in this study because about 80 per cent of the nitrogen excreted by birds is in the form of uric acid (30). It was felt that this extensive synthesis of purine would favor the experimental elucidation of the chemical mechanisms involved.

## EXPERIMENTAL

The animals were maintained on an adequate diet (see below) at approximately constant weight and given about 15 per cent of the total dietary nitrogen in the form of nitrogen of the compound under investigation. For the rats the compound was mixed with the food; for the pigeons it was administered every 6 hours in aqueous solution by means of rubber tubing

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† Died, September 11, 1941.

passed into the crop. All feeding experiments were carried out over a 3 to 4 day period.

It has been shown (31, 32) that ammonia nitrogen administered to rats extensively replaces the nitrogen in a large number of body constituents. In Experiment 1 the isotopic nitrogen in the form of ammonium citrate, prepared as described by Schoenheimer and Ratner (33), was administered to three pigeons. Uric acid was isolated from the combined excreta of the last 4 days of the experiment. Amino acids and purines were isolated, after removal of the non-protein nitrogen by trichloroacetic acid, from the pooled internal organs of the three birds.

TABLE I

*Experiment 1. Feeding of Isotopic Ammonium Citrate to Pigeons*

Number of pigeons, three; nitrogen fed as isotopic ammonium citrate per pigeon per day, 50 mg; duration of isotope administration, 4½ days, concentration of isotope, 1.13 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$	Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in ammonium citrate given
Mixed purines of internal organs . . .	0.067	5.9
Guanine	0.071	6.3
Adenine	0.062	5.5
Uric acid (last 4 days)	0.175	15.5
Amide nitrogen	0.070	6.2
Glutamic acid	0.023	2.0
Aspartic "	0.031	2.7
Arginine . .	0.008	0.7
Ornithine portion	0.001	0.1
Amidine portion	0.005	0.4
Histidine	0.007	0.6

As shown in Experiment 1 (Table I), isotopic nitrogen was found in high concentration in the uric acid isolated from the excreta of the last 4 days of the experiment. The  $N^{15}$  levels found here in uric acid are comparable to those for urea in the rat after ammonia feeding (31, 32). The ready conversion of ammonia nitrogen to uric acid suggests that ammonia is directly utilized for uric acid production in pigeons.

Glutamic acid and aspartic acid had much lower isotope levels by comparison, but, as in the rat, these were still high. This suggests that amino acid nitrogen of protein in pigeons is subject to the same type of rapid replacement by other body nitrogen as shown in the case of the rat, and also that the concept of a generally available metabolic nitrogen pool (34) may be applied to the pigeon.

The mixed purines of the internal organs contained a concentration of  $N^{15}$  that was 3 times that of the glutamic acid isolated from the proteins of these organs. It was higher than one-third of the concentration of the isotope in the excreted uric acid. The guanine and adenine separately isolated from this same source showed similar concentrations. These data indicate that a close relationship exists between the cellular purines of the organs and the production of uric acid by the pigeon. They suggest that if there are two pathways leading respectively from ammonia nitrogen to cellular purines and from ammonia nitrogen to uric acid these have some portion of each in common. It is noteworthy that the "amide nitrogen" of the proteins of the internal organs had the same isotope level as the purines.

Arginine isolated from the proteins of the internal organs took up no significant amount of  $N^{15}$ , a finding consonant with the dietary indispens-

TABLE II

*Experiment 2. Feeding of Isotopic Urea to Pigeons*

Number of pigeons, two; nitrogen fed as isotopic urea per pigeon per day, 50 mg.; duration of isotope administration, 4 days; concentration of isotope, 1.76 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$	Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in urea given
Mixed purines	0.004	0.2
Uric acid	0.034	1.9

ability of arginine for birds (35) and with the absence of arginase in bird liver (36). Almost no  $N^{15}$  replaced the nitrogen of histidine also isolated from the organ proteins. Since Schoenheimer and coworkers have shown that isotopically labeled amino acids replace the corresponding amino acids in proteins to a very marked extent, it is safe to conclude that the synthesis in pigeons of purines and of uric acid from orally administered ammonia does not involve incorporation of this ammonia nitrogen in either protein or non-protein forms of arginine or histidine as intermediary substances. Moreover, if either of these amino acids independently furnished nitrogen for purine synthesis, the actual  $N^{15}$  concentration in that portion of the molecule replaced by ammonia nitrogen would have reached an even higher isotope content than is indicated by our analysis of the whole purine molecule.

In order to obtain direct evidence of the rôle of urea in purine synthesis, urea-containing  $N^{15}$  was fed to pigeons in Experiment 2 (Table II). Uric acid was again isolated from the excretions and mixed purines were isolated from the proteins of the internal organs. The mixed purines

showed no replacement by isotopic nitrogen and the concentration of  $N^{15}$  in the uric acid was so low, in comparison with the levels reached after ammonia feeding, as to preclude the likelihood that urea is normally an intermediary substance in purine synthesis in the bird.

In Experiment 3, feeding of isotopic ammonia to pigeons was repeated and the organs were treated separately in an attempt to discover which organs contribute to the high isotope level found in the purines isolated in Experiment 1. The purines were isolated as the mixed cuprous compounds of adenine and guanine. From the work of Graff and Maculla (37) on the accuracy of the  $Cu_2O$  estimation of purines, and from the close correspond-

TABLE III

*Experiment 3. Feeding of Isotopic Ammonium Citrate to Pigeons*

Number of pigeons, three, nitrogen fed as isotopic ammonium citrate per pigeon per day, 50 mg.; duration of isotope administration, 4½ days; concentration of isotope, 1.31 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$	Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in ammonium citrate given
Mixed purines of		
Lungs	0.017	1.5
Heart	0.016	1.4
Blood	0.013	1.1
Pancreas	0.042	3.7
Kidneys	0.044	3.9
Gonads	0.062	4.7
Gastrointestinal tract	0.080	7.1
Liver	0.101	8.9
Uric acid excreted on last day	0.239	21.1

ence in isotope concentration between the mixed cuprous purines and the isolated individual purines when both were analyzed, we may conclude that the average  $N^{15}$  content of the two purines, adenine and guanine, is accurately measured by analysis of the mixed cuprous precipitate. The data (Table III) clearly show that the liver was the organ most extensively involved in the nitrogen replacement in purines. They do not prove directly that purine synthesis takes place in other organs, since labeled purines could have been transported in the blood. However, the fact that the isotope level in the blood purines was lower than in any of the other organs examined argues against transportation. All organs may take up nitrogen, though to varying degrees. Large amounts of isotope were also acquired by purines of the gastrointestinal tract, gonads, pancreas, and kidneys.

Only small amounts of  $N^{15}$  were found in the purine nitrogen of lung, heart, and blood. The work of Minkowski (19) on removal of the liver in geese indicates that this organ is essential for uric acid formation. That it may not be the only organ so involved, however, is evident from the work of Schuler and Reindel (38).

In Experiment 1, the isotopic uric acid was secured from the mixed excretions of the last 4 days. In Experiment 3 the uric acid was that excreted on the last day only and, therefore, had an even higher  $N^{15}$  content than in Experiment 1.

In Experiment 4 (Table IV) with ammonium citrate, a higher concentration of isotope was fed to pigeons in a similar manner. This time the nucleic acid of the internal organs was isolated and from this the individual

TABLE IV

*Experiment 4. Feeding of Isotopic Ammonium Citrate to Pigeons*

Number of pigeons, four; nitrogen fed as isotopic ammonium citrate per pigeon per day, 50 mg.; duration of isotope administration, 3 days; concentration of isotope, 4.51 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$	Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in ammonium citrate given
Nucleic acid of internal organs	0.107	2.4
Mixed purines	0.108	2.4
Guanine	0.139	3.1
Xanthine (derived from guanine)	0.126	2.8
Uric acid (last day)	0.399	8.8
Creatinine from carcass	0.007	0.16
Adenylic acid of muscle	0.031	0.69

purines. In this way the previous results could be checked at a different isotope level and the extent of nitrogen replacement in nucleic acid purines could be investigated.

As in Experiment 1, guanine of the internal organs took up a large amount of isotope, the value again reaching a large fraction (about one-third) of that observed in the excreted uric acid. To determine whether the isotope was present in the purine ring or only in the substituent imino group, the guanine isolated was decomposed and the resulting xanthine isolated. As the latter contained almost as much isotope as the guanine from which it was derived, it is clear that both the ring and the substituent amino group are involved in the uptake of ammonia nitrogen.

The isotope concentration in the crude nucleic acid was the same as that in the cuprous precipitate of mixed purines isolated from the internal organs.

This means that pyrimidine nitrogen is replaced by  $N^{15}$  to an extent about equal to the replacement in purines.

Creatine of the carcass isolated<sup>1</sup> as creatinine showed no significant uptake of isotope. Adenylic acid was isolated<sup>2</sup> from the pigeon breast muscle and showed only a relatively slight uptake of  $N^{15}$ .

Experiment 5 was performed in the same way as Experiment 4 and with almost the same concentration of  $N^{15}$ , except that more pigeons were used in an attempt to gain enough nucleic acid to permit isolation of pyrimidines and of hypoxanthine by decomposition of adenine. The latter took up about the now expected amount of  $N^{15}$  (Table V). The hypoxanthine isolated after decomposition of the adenine had an isotope content of 0.140 atom per cent excess  $N^{15}$ . It can be calculated from this figure and that of the starting adenine that the amino group of adenine at position 6 had

TABLE V

*Experiment 5. Feeding of Isotopic Ammonium Citrate to Pigeons*

Number of pigeons, six, nitrogen fed as isotopic ammonium citrate per pigeon per day, 50 mg.; duration of isotope administration, 3 days; concentration of isotope, 4.42 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$	Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in ammonium citrate given
Nucleic acid of internal organs	0.111	2.5
Adenine	0.118	2.7
Hypoxanthine (derived from adenine)	0.140	3.2
Cytosine	0.137	3.1
Thymine	0.132	3.0

an isotope content of only 0.030 atom per cent excess. It is to be noted that the isotope value for hypoxanthine was the same as that for guanine in the entirely comparable Experiment 4. The substituent amino group of adenine is therefore apparently only slightly involved in nitrogen replacement in the synthesis of adenine from ammonia nitrogen, whereas that of guanine takes full part in the synthesis of the latter. Support for this conjecture is found in Experiment 1 where, within the limits of error of the experimental method, guanine contained about 15 per cent more isotope than did adenine.

In Experiment 5 the pyrimidines were also isolated, although thymine could not be obtained as pure as desired. Approximately as much isotope was taken up by thymine and also by cytosine as by the purines, showing

<sup>1</sup> Isolated by A. Plentl of this Department.

<sup>2</sup> Isolated by H. M. Kalckar of this Department.

that the statements made above regarding the rapid rate of synthesis of purines from ammonia nitrogen and the rôle of these compounds in uric acid formation apply also to the pyrimidines of the nucleic acids in the internal organs of pigeons.

Experiment 6 (Table VI) was undertaken to determine whether or not the same facts hold true for rats. The animals were kept on an adequate diet (see below) and at approximately constant weight, the isotopic am-

TABLE VI

*Experiment 6. Feeding of Isotopic Ammonium Citrate to Rats*

Number of rats, two in Group A, two in Group B; nitrogen fed as isotopic ammonium citrate per rat per day, 40 mg., duration of isotope administration, 3 days; concentration of isotope, 4.51 atom per cent excess  $N^{15}$ .

	Atom per cent excess $N^{15}$		Atom per cent $N^{15}$ calculated on basis of 100 per cent $N^{15}$ in ammonium citrate given	
	Group A	Group B	Group A	Group B
Nucleic acid of internal organs	0.045	0.056	1.0	1.2
Mixed purines	0.051	0.055	1.1	1.2
Glutamic acid	} of mixed in- "Amide" nitro-} ternal or- gen } gans	0.086	1.9	
Urea			1.4	
Allantoin	0.565	0.702	13.0	16.0
Mixed pyrimidines	0.058	0.069	1.3	1.5
Guanine	0.074	0.114	1.6	2.5
	0.054	0.058	1.2	1.3
Combined Groups A and B				
Guanine	0.063		1.4	
Xanthine (derived from guan- ine)	0.050		1.1	
Adenine	0.051		1.1	
Hypoxanthine (derived from adenine)	0.049		1.1	

monium citrate being mixed with the food. The experiment was performed in duplicate with two rats for each part. Where not enough material could be obtained from each replicate, the two were combined. The nucleic acid of the internal organs was isolated as the sodium salt and from this the purines and pyrimidines were then isolated. Since not enough material was present to permit all the steps found necessary to obtain analytically pure cytosine and thymine, the pyrimidines of each experiment were isolated together as the silver complexes (39). It was found that the purines and pyrimidines had taken up isotope in equally large amounts.



Confirmation of the rapid replacement of pyrimidine nitrogen by ammonia nitrogen was found in the  $N^{15}$  content of the nucleic acid of each duplicate experiment. As with the pigeon experiments, the guanine combined from both experiments had a higher  $N^{15}$  content than that of combined adenine by about 20 per cent. Again, as in the pigeons, the isotope was in the ring portion of both purine molecules. In guanine it was present in greater concentration in the substituent imino group, as shown by analysis of the xanthine obtained by decomposition of the guanine. However, in adenine the nitrogen isotope appeared to be distributed almost equally between the ring portion of the molecule and the substituent group. As with the pigeons, "amide" nitrogen had an  $N^{15}$  content about the same as that of the purines and pyrimidines. Glutamic acid was about 30 per cent higher. Urea was the chief excretory form of  $N^{15}$ . The isotope concentration in allantoin was about the same as that in the purines, a finding which supports the well founded concept that allantoin is the end-product of purine metabolism in the rat. Attention is drawn to the observation that the  $N^{15}$  level of glutamic acid nitrogen was higher than that of the mixed organ purines and pyrimidines in the rat, whereas the reverse was true in the pigeon (Table I).

### Methods

*Isolation of Mixed Cuprous Purines*—In Experiments 1, 2, and 3, after the birds had been killed by a blow or by ether, the internal organs were removed, the gastrointestinal tracts opened and well washed out with water, and the organs placed immediately in cold 6 per cent trichloroacetic acid. The organs so treated were heart, lungs, liver, spleen, pancreas, kidneys, gonads, and gastrointestinal tract. The residue, after the trichloroacetic acid was filtered off, was treated essentially by the method of Graff and Maculla (37) for quantitative estimation of purines in body fluids.

After the removal of a sample of the final cuprous purine precipitate for isotope analysis, the precipitate was suspended as finely as possible in water which was made acid to Congo red paper with HCl, and  $H_2S$  was passed in. After filtration and expulsion of the  $H_2S$ , the resulting solution was evaporated to a volume of about 10 to 15 ml. and was ready for purine isolation.

*Isolation of Nucleic Acid Salt*—In Experiments 4, 5, and 6, nucleic acid was first isolated as a sodium salt; then the various bases were isolated from this. The method consisted of extraction of the non-autolyzed, fat-free tissues by 10 per cent NaCl at 70° and precipitation of the nucleic acid as a sodium salt by the addition of 3 volumes of 95 per cent ethanol. Through the extraction with 10 per cent NaCl the method corresponded chiefly to that of Clarke and Schryver (40).

The animals were anesthetized with ether, as much blood as possible was removed immediately by cardiac puncture, and the internal organs (liver, kidneys, gonads, spleen, pancreas, gastrointestinal tract, heart, and lungs) were taken out without delay, rapidly washed, and placed in solid carbon dioxide. The gastrointestinal tracts were first opened and their contents well washed out. In Experiment 4 the heart and lungs were not included and in Experiment 6 the seminal vesicles of the rats were included.

The organs were ground up with solid CO<sub>2</sub> in a chilled iron mortar, put immediately into acetone, allowed to stand 24 hours, filtered by suction, and replaced in fresh acetone for another 24 hours. After filtration, the same extraction was performed twice with ethanol-ether (2:1). The final residue was washed twice with ether, sucked very dry, and all traces of solvent removed in a desiccator under constant suction for 3 to 4 hours. The tissues were then ground more finely in a mortar and placed in boiling 10 per cent NaCl (20 cc. per gm. of the dried tissue). After 10 to 15 minutes boiling, the suspension was stirred mechanically on a steam bath for 18 hours, the volume being kept constant by frequent additions of water.

The suspension was again heated to boiling, filtered by gentle suction through coarse filter paper on a steam-heated Buchner funnel, and washed once with about 0.05 volume of hot 10 per cent NaCl (Filtrate 1). The residue was again extracted with about one-third the original amount of 10 per cent NaCl in the same manner for about 8 hours, filtered, and washed three times with 0.05 volume of hot 10 per cent NaCl (Filtrate 2). Further extractions yielded no significant amounts of nucleic acid.

Filtrates 1 and 2 were then combined and the following procedure<sup>3</sup> was followed. 3 volumes of 95 per cent ethanol were slowly added with stirring and the suspension set in the ice box overnight. The flocculent, white precipitate which settled out was centrifuged down and washed twice with 65 per cent ethanol, twice with 95 per cent ethanol, once with ether, and finally was air-dried by suction. It consisted of a fine white powder, not at all sticky. This nucleic acid salt without further purification was used for isolation of nuclein bases.

In Experiments 4 and 6, the nucleic acid so obtained was hydrolyzed by the formic-HCl method, preparatory to Cu<sub>2</sub>O precipitation, as described above. In Experiment 5, Levene's method (42) of acid hydrolysis in methyl alcohol was used.

*Isolation of the Various Bases of Nucleic Acid*—Guanine was isolated essentially by the method of Levene (42), the base being finally obtained, however, as the crystalline sulfate.

<sup>3</sup> Suggested by Dr. Samuel Graff as an adaptation of parts of several other methods (chiefly Jorpes (41)).

*Analysis*—Dried over 5.6 M sulfuric acid at room temperature without vacuum

		Per cent N calculated	Per cent N found
Experiment 1	$C_5H_5ON_5$	46.4	43.9
"	4. $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot 2H_2O$	32.1	31.8, 31.8
"	6, A.	32.1	32.1
"	6, B.	32.1	31.7

Adenine was isolated as the picrate by the method of Levene (42).

		Per cent N calculated	Per cent N found
Experiment 1.	$C_5H_5N_5 \cdot C_6H_3O_7N_3$	30.77	28.13
"	6 (A and B combined). $C_5H_5N_5 \cdot C_6H_3O_7N_3$	30.77	30.12
Experiment 5.	$C_5H_5N_5 \cdot C_6H_3O_7N_3$	30.77	30.48

For the isolation of pyrimidines in Experiment 5 the following method was found more suitable than any other tried. Purines were removed by hydrolyzing 3.0 gm. of nucleic acid according to the methyl alcohol method of Levene (see above). After removal of both crops of purine hydrochloride by centrifugation, the supernatant was evaporated to a dark syrup which was taken up in 25 ml. of 20 per cent HCl and heated at 175° in a bomb tube for 2 hours. The mixture was diluted with water to 200 ml., brought to a boil, and filtered. The filtrate was evaporated to dryness *in vacuo* and the residue taken up in about 200 ml. of water. Solid silver carbonate was added until all chloride was precipitated, the solution being kept slightly acid to Congo red by addition of nitric acid. The suspension was filtered by suction through diatomaceous earth, the excess silver removed with a minimal amount of HCl, and the filtrate evaporated to about 4 ml. Crystals of thymine came out slowly in the ice box over a period of 2 days, were filtered off by suction, and washed with water. They were twice recrystallized from water, a small amount of charcoal being used for complete decolorization. They were dried in a desiccator *in vacuo* over phosphorus pentoxide at room temperature. Yield 13.0 mg.

*Analysis*—Kjeldahl method Experiment 5

$C_5H_5O_2N_2$ . Calculated, N 22.2, found, N 24.4

For isolation of cytosine the filtrate from the crystals of thymine was brought to a pH acid to litmus and alkaline to Congo red, boiled with a little charcoal, and filtered. The filtrate was evaporated to a volume of about 10 ml. and an excess of 5 per cent picric acid in 95 per cent ethanol added. The mixture was placed in the ice box; after 18 hours the precipitate was filtered off by suction and washed well with cold water. The precipitate of cytosine picrate was recrystallized from water twice, and the crystals dried at 105° for 3 hours *in vacuo* over  $P_2O_5$ . Yield 44.0 mg.

*Analysis*—Dumas method. Experiment 5

$C_4H_5ON_3 \cdot C_6H_3O_7N_3$ . Calculated, N 24.74, found, N 26.67

In an effort to obtain pure cytosine, the free base, the picrate was taken up in 1.0 N HCl and the picric acid extracted continuously with ether. The aqueous solution was then treated with a stoichiometric amount of dilute  $\text{H}_2\text{SO}_4$  (0.12 milliequivalent), evaporated almost to dryness, and placed in a desiccator *in vacuo* over NaOH. The dry crystalline residue was moistened with water and replaced in the desiccator as before in order to remove all traces of HCl. The crystals were then taken up in water, filtered, and evaporated to a volume of 0.5 ml. Addition of 3 volumes of 95 per cent ethanol resulted in a crystalline, white precipitate. The crystals were left in the ice box overnight, filtered off by suction, and washed twice with 75 per cent ethanol. The product, 18 mg., was recrystallized from 75 per cent ethanol and taken up in water. The solution was neutralized to litmus by careful addition of NaOH and evaporated to small volume (about 0.5 ml.), when mother-of-pearl plates separated. The mixture was allowed to stand in the ice box overnight; the product, 5.3 mg., was filtered off by suction, recrystallized from water, and dried at  $105^\circ$  *in vacuo* over phosphorus pentoxide for 2 hours. Yield about 4 mg.

Experiment 6  $\text{C}_4\text{H}_5\text{ON}_3$  Calculated, N 37.8; found (Kjeldahl), N 37.2

For both parts of Experiment 6, the pyrimidines were isolated as the mixed silver-pyrimidine complexes according to the method of Kossel (39). For this, hydrolysis was performed at  $175^\circ$  in 20 per cent HCl, as above. In Experiment 6, A the hydrolysate, after removal of HCl by evaporation, was made just acid to Congo red by addition of NaOH; then a slight excess of silver nitrate was added and the precipitate filtered through diatomaceous earth. Saturated  $\text{Ba}(\text{OH})_2$  was added until the reaction of the suspension was slightly alkaline to Congo red. After filtration through kieselguhr and charcoal, to the colorless filtrate more silver nitrate was added until an immediate brown precipitate was produced when a drop of the solution was made alkaline with  $\text{Ba}(\text{OH})_2$ . Saturated  $\text{Ba}(\text{OH})_2$  solution was now added slowly until a lasting, definitely brown precipitate formed. This was centrifuged off at once, washed plentifully with water, taken up in water, and subjected to a stream of  $\text{H}_2\text{S}$ . After removal of the sulfide by filtration with charcoal, the resulting clear solution was in part submitted for isotope analysis. The yield, as measured by Kjeldahl analysis, was 11 mg. of nitrogen, corresponding to about 35 mg. of pyrimidine base. In Experiment 6, B the same procedure was followed except that an attempt was made to isolate the individual pyrimidines after removal of silver sulfide. Only about 2 mg. of each could be isolated and, as purification or identification was impossible with such amounts, it was decided to make an isotope analysis on the total mixed product of silver precipitation as in Experiment 6, A.

*Isolation of "Amide" Nitrogen, Aspartic and Glutamic Acids, Histidine,*

*Arginine*—These were all isolated from the residues of the internal organs which had previously been treated with trichloroacetic acid and then either hydrolyzed for purine removal by the cuprous oxide method or extracted with 10 per cent NaCl to obtain nucleic acid. In either case, after the ensuing protein hydrolysis with 20 per cent HCl, it was found necessary to remove the amino acids from the contaminating salts by drying the residue *in vacuo* and extracting with 95 per cent ethanol. The methods used were those previously described from this laboratory (43, 31). The histidine, isolated as the monohydrochloride, was finally analyzed as the 3,4-dichlorobenzenesulfonate (44).

	Yield mg	N calculated	N found (Kjeldahl)
Glutamic acid hydrochloride	268	7.6	7.6
Same	209	7.6	7.5
Histidine 3,4-dichlorobenzenesulfonate	9	6.9	6.8, 6.8
Aspartic acid	107	10.5	10.4, 10.4

*Decomposition of Arginine to Ornithine*—Ornithine was obtained as follows: About 350 mg. of arginine flavianate were dissolved in 35 ml. of concentrated HCl and chilled in the ice box; the flavianic acid was filtered on a sintered glass funnel and washed well with cold concentrated HCl. The slightly yellow filtrate was diluted with about 2 volumes of water, boiled with charcoal, and filtered hot. After evaporation to about 30 ml. the clear, colorless solution was treated with 5 gm. of solid BaO and boiled in a stream of nitrogen gas (45) with the use of a reflux condenser and acid trap. After 24 hours, 95 per cent of the calculated quantity of  $\text{NH}_3$  had evolved. The barium was removed by means of  $\text{H}_2\text{SO}_4$  and isotope analyses were made on the filtrate and on the ammonia.

*Isolation of Uric Acid*—The method used was essentially that described by Neubauer and Huppert (46). The droppings of the pigeons, collected over a given period, were mixed together and extracted continuously with a solution of 85 per cent alcohol containing acetic acid in 10 per cent concentration for 10 hours. After being allowed to dry, the undissolved residue was ground up, heated on a steam bath for a few minutes in about 3 volumes of 2 per cent NaOH, cooled, centrifuged, and washed with about 1 volume of 2 per cent NaOH. The combined supernatants were then immediately saturated with  $\text{CO}_2$  (about  $\frac{1}{2}$  hour) and let stand overnight. The colorless precipitate of sodium urate was centrifuged off, washed once with dilute  $\text{NaHCO}_3$  solution, suspended in water, and strongly acidified, with mechanical stirring. The resulting uric acid was centrifuged, washed with water, and dried. It was then taken up in concentrated  $\text{H}_2\text{SO}_4$  at 20–25° and filtered by suction through asbestos. The filtrate was slowly diluted to about 50 per cent; the resulting precipitate was centrifuged and taken up

in water with just enough NaOH; the solution was warmed for a moment with charcoal, filtered, and promptly made acid to Congo red with HCl. The material was permitted to stand in the ice box overnight and after filtration by suction the product was recrystallized from dilute NaOH and HCl with charcoal as many as three to four times until crystalline, white material separated. The uric acid was dried at 100° over  $P_2O_5$  *in vacuo* and analyzed by the Kjeldahl method. The N content in four experiments ranged from 32.9 to 33.3 (calculated 33.3).

*Decomposition of Guanine to Xanthine*—The method was based on those of Strecker (47) and of Fischer (48). To 28 mg. of guanine were added 55 mg. of concentrated  $H_2SO_4$  in 0.4 ml. of water. With the reaction tube just within the mouth of the steam bath, 21 mg. of  $NaNO_2$  were added slowly in a minimal amount of  $H_2O$  with much stirring. After being allowed to stand in the ice box overnight, the suspension was centrifuged. The orange precipitate was washed with cold water and dissolved by warming in a minimal amount of 15 per cent  $H_2SO_4$ . This solution was diluted with hot water to 2 per cent, decolorized with charcoal, and evaporated to a small volume under a current of air at 75°. It was cooled slowly in the room (one may have to dilute slightly if crystals do not form), and then in the ice box. The crystals were centrifuged, washed twice with cold 15 per cent  $H_2SO_4$ , and recrystallized from 15 per cent  $H_2SO_4$  in the same way. Finally the product was taken up in water, neutralized with NaOH, and allowed to stand in the ice box overnight. The precipitate was filtered by suction, washed well with water, and dried at 140° over  $P_2O_5$  *in vacuo* for 2 hours. The yields of xanthine (calculated, N 36.8) were 14 and 5 mg. from 38 and 28 mg. respectively of guanine. Found, N 36.2, 36.0.

*Decomposition of Adenine to Hypoxanthine*—The method followed was in part that of Kossel (1). To a warm solution of 51 mg. of adenine in 1.4 ml. of 7 per cent  $H_2SO_4$  were added slowly, with stirring, 80 mg. of  $NaNO_2$  dissolved in a minimal amount of water. The tube was then placed in a boiling water bath for 3 to 5 minutes. When cool, the solution was made alkaline to Congo red but acid to litmus and cooled in the ice box overnight. The precipitate was then centrifuged off, washed twice with cold water, taken up in a small volume of 3 per cent HCl, filtered hot, evaporated to a small volume, and treated with an excess of gold trichloride (49). The solution was cooled in the ice box overnight, and the thick orange crystals were filtered off, washed well with 1 per cent HCl, and recrystallized from 3 per cent HCl. The yields of the aurichloride,  $C_5H_5ON_4Cl_4Au$  (calculated, N 11.8), dried under atmospheric pressure over  $P_2O_5$ , were 77 and 18 mg. from 51 and 8 mg. respectively of adenine. Found N 11.7, 11.8.

*Isolation of Urea (Rat; Experiment 6)*—The method followed was that of Graff, Maculla, and Graff (50) with slight modifications personally sug-

gested by those authors. The dioxanthylurea gave correct N values (6.6) by Kjeldahl analysis.

*Isolation of Allantoin*—The method used was that of Wiechowsky (51). It was found difficult to isolate pure samples of allantoin and also to digest them completely for Kjeldahl analysis. The samples were dried *in vacuo* at 125° for 2 hours over P<sub>2</sub>O<sub>5</sub>. One sample was found to contain 3 per cent inorganic ash; when this was allowed for, the sample gave a satisfactory analysis. It was necessary to digest for 8 hours by the Kjeldahl procedure and to make several additions of H<sub>2</sub>O<sub>2</sub> during the digestion to get satisfactory values for nitrogen on any of the samples.

C <sub>4</sub> H <sub>6</sub> O <sub>2</sub> N <sub>4</sub> .	Calculated.	C 30.4, H 3.8, N 35.5
	Found.	" 30.4, " 3.9, " 35.4, 35.7

*Diets*—The rats were kept on an adequate stock diet (32). The pigeons were kept on an adequate diet of mixed grains, of which a sample by Kjeldahl analysis had a nitrogen content of 1.86 per cent. The pigeons ate this diet without restriction.

#### SUMMARY

These experiments demonstrate that ammonia nitrogen in pigeons and rats is rapidly incorporated into purines and pyrimidines of the nucleic acids of the internal organs, and also into uric acid and allantoin for excretion. Purines and pyrimidines accordingly belong to the large number of body substances whose nitrogen is being rapidly replaced at all times. Replacement of nitrogen in these compounds must mean that the ring structure is broken in the process; so that we have here another instance in which complex compounds in the body are not relatively static but are undergoing constant breaking of carbon-nitrogen, and perhaps other, linkages. In both the rat and the pigeon the substituent imino group in guanine undergoes a greater replacement by isotope than does that of adenine.

In the pigeon the rates of excretion and the relative concentrations suggest that nitrogen traversing the pathway of synthesis from ammonia to uric acid passes through purines and pyrimidines of at least some of the cells of the internal organs, and that these substances, as intrinsic chemical constituents of the cell, are also intermediaries in the conversion of body nitrogen to purine for excretion. In the rat the same appears to be true, although a much smaller proportion of the general body nitrogen is converted by way of the purines and pyrimidines to the end-product, allantoin.

It should be noted that nitrogen fed as ammonia to birds did not find its way into muscle creatinine and entered to only a small extent into muscle adenylic acid.

The fact that the isotope levels found in the pyrimidines are approxi-

mately the same as those in the purines throws further light on the part played in metabolism by pyrimidines. Evidently these substances undergo nitrogen replacement in the same manner as do the purines. The isotope levels are so closely alike as to suggest that pyrimidines are involved in the same metabolic processes as are the purines. Finally, if they are converted to a purine for excretion, as the data indicate, it is quite possible that they are synthesized into body purines as well.

Two earlier observations enable us to extend the significance of these findings. Firstly, Minkowski (19) noted that after removal of the livers from geese the uric acid of the urine was replaced by ammonia and lactic acid, and this fact, taken together with the present demonstration that ammonia nitrogen is rapidly incorporated into purines and pyrimidines, makes possible the conclusion that ammonia itself, or some closely related substance, is a normal intermediate in purine and pyrimidine synthesis in the bird. The similar results with the rat suggest that the same is true in this animal. These findings, which agree with earlier evidence regarding the conversion of ammonia nitrogen to purines (15-17), support current views (32, 36, 52) as to the importance of ammonia in the general nitrogen economy of the animal organism.

Secondly, Schoenheimer and his collaborators have shown that in the rat ammonia nitrogen is rapidly converted into amino acids and urea and apparently enters a generally available, biologically labile "pool" of nitrogen which is involved in numerous interconversions in the body. We now see that the same general situation apparently also obtains in the pigeon. Since ammonia nitrogen becomes indistinguishably merged with the general metabolic nitrogen which is made up in large part of labile protein nitrogen of both food and body, it is evident that protein nitrogen must be continually used for purine and pyrimidine synthesis. This fits well with the fact that almost all of the nitrogen excreted by the pigeon is in the form of uric acid, and with the fact that in all animals so far tested the excretion of purines is not reduced by restricting the dietary nitrogen to protein sources. Furthermore, it corroborates the conversion of amino acids to purines, observed in numerous feeding and tissue slice experiments.

As for other specific precursors the data given here show that the pigeon is incapable of utilizing urea for purine synthesis. Furthermore, in this bird, arginine and histidine do not lie on the pathway of the synthesis of purine and pyrimidine from ammonia. The absence of isotope in arginine testifies to the essential character of this amino acid in bird nutrition. As for the rat, since the uptake of  $N^{15}$  in histidine isolated from the carcass proteins after ammonia feeding is confined to the  $\alpha$ -amino group (53), the imidazole nitrogen takes no part in purine synthesis from ammonia. In the case of arginine in the rat, although ammonia nitrogen is rapidly in-



incorporated into the amidine group (31), little or no isotope could be found in urinary allantoin after the feeding of arginine marked with  $N^{15}$  in the amidine group (54). Although these three substances, urea, histidine, and arginine, could (except for urea in the pigeon) conceivably contribute nitrogen to some part of the purine or pyrimidine molecule not built up from ammonia nitrogen, it seems improbable that they are specific precursors of purines or pyrimidines in either animal.

The ready synthesis of nucleic acids from general metabolic nitrogen in rats and pigeons suggests that this phenomenon is wide-spread in the animal kingdom. It appears possible that in such species as excrete nitrogen in the form of a purine there has not developed a different type of metabolic pathway but that a wide-spread biological process has been adapted to special purposes.

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# QUANTITATIVE MICRODETERMINATION OF AMMONIA IN THE PRESENCE OF GLUTAMINE AND OTHER LABILE SUBSTANCES

By REGINALD M. ARCHIBALD\*

*(From the Hospital of The Rockefeller Institute for Medical Research, New York)*

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While many forms of apparatus and methods have been devised for the quantitative microdetermination of  $\text{NH}_3$ , we were able to find in the literature none which was entirely suitable for use in the presence of substances, such as glutamine, which are labile at a pH higher than 10.0 and which are also thermolabile.

The technique for "direct" nesslerization of filtrates obtained from precipitation of proteins outlined by Gentzkow (1), in which gluconate and persulfate are added to prevent clouding of the Nessler's solution, is a distinct advance, but the high pH of Nessler's reagent results in relatively rapid increase in ammonia content of the unknown when labile substances such as glutamine are present. Conway's (2, 3) micro diffusion technique is useful under many circumstances, but the saturated potassium carbonate solution used to liberate the ammonia and the length of time required to effect transfer involve considerable error due again to relatively high "blank" values from destruction of labile glutamine. While reproducible and accurate results seem to be obtained by Conway (3) and Koprowski (4) with the more rapid incomplete diffusion method, in which only 40 to 60 per cent of the ammonia is transferred and measured, even here the unknown is exposed to high pH for an appreciable time and many "units" are required to obtain comparable results for a series of moderate size.

The technique outlined below has been found satisfactory for the determination of ammonia in biological preparations (as for example blood plasma) in which the concentration of labile ammonia-forming material is important in comparison with the free ammonia. It has proved useful also in studying the influence of various factors on the rate at which glutaminase hydrolyzes ammonia from glutamine. In such a study the concentration of ammonia to be measured is small compared with the concentration of residual labile substrate and the use of a method which involves gentle treatment is imperative. The method has proved so simple and rapid that it has been preferred in this laboratory for micro distillation and determination of ammonia even in the absence of labile ammonia-forming material,

\* Fellow of the National Research Council, Division of Medical Sciences.

although under these circumstances the method offers fewer advantages over ones already described.

Because of the mild conditions used, the technique can be applied readily to the removal and quantitative determination of relatively large amounts of preformed ammonia followed by the quantitative determination of minute amounts of ammonia from labile substances in the same aliquot, as in the successive determination of preformed ammonia and glutamine amide N in normal urine.

The method in principle is identical with that described by Pucher *et al.* (5) but has been modified to apply to blood and to smaller portions of ammonia and to facilitate rapid transfer of distillate and washings to the volumetric container in which it is nesslerized.

*Reagents—*

$H_2SO_4$ , approximately 0.04 N. 0.50 cc. of concentrated  $H_2SO_4$  diluted to 500 cc. The solution is stored in a container through the stopper of which passes a 5 cc. Mohr pipette.

*Saturated borate buffer of pH 10.1.* Borax, U.S.P., powder 188 gm., and 47 cc. of 18 N NaOH (or 34 gm. of NaOH) are boiled with 1900 cc. of water 15 minutes to remove traces of ammonia. The solution is then allowed to cool to 30° and the volume made up to 2 liters. On being cooled to room temperature some crystals form but the supernatant has a pH of 10.0 to 10.1. A portion is stored in a convenient sized container the stopper of which is fitted with a 2 cc. Mohr pipette.

*Antifoam.* Equal parts of tributyl citrate<sup>1</sup> and methyl benzoate<sup>2</sup> in a dropping bottle.

*Distilled water* should have a low ammonia content. Redistillation has not proved necessary in our laboratory.

*Standard  $(NH_4)_2SO_4$ , 0.0005 M.* 1 cc. contains 0.014008 mg. of N.

*Nessler's solution* as prepared by Koch and McMeekin (6).

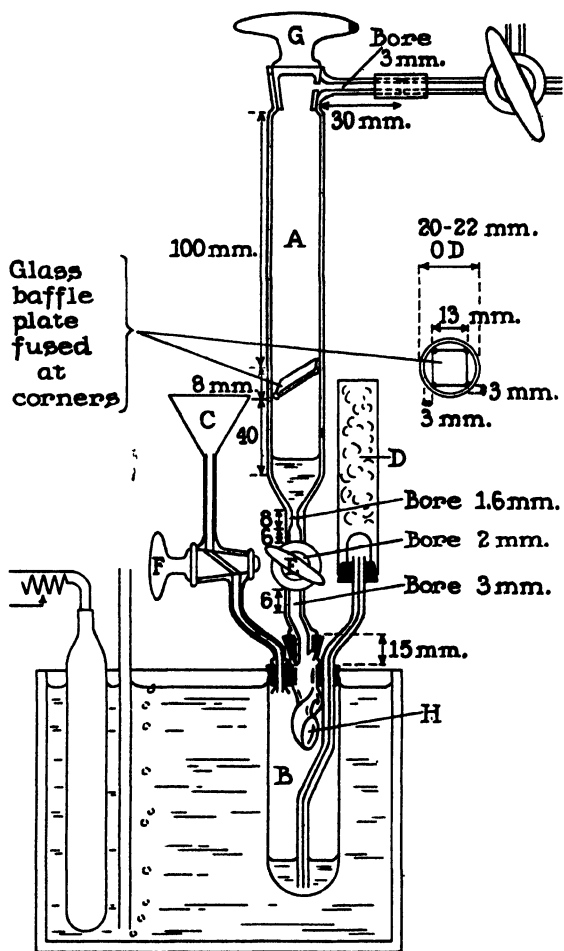
*Method*

The distillation apparatus is shown in Fig. 1. A 150 watt hot-point heater in series with a variable resistance maintains the temperature in the outside bath at 50° ( $\pm 0.5^\circ$ ) without the aid of a thermostat. The bath is stirred by a current of air bubbles. The receiving unit A (prepared according to our specification by Machlett and Son) is charged with 5 cc. of 0.04 N  $H_2SO_4$ . The stop-cock of this unit should be kept lightly greased with a heavy rubber grease. Lighter grades of lubrication result in cloudy distillates. The ground glass stopper G is not greased and remains turned so as to connect the chamber to the side arm. With the unit A in

<sup>1</sup> Commercial Solvents Corporation, 17 East 42nd Street, New York.

<sup>2</sup> Givaudan Delawanna, Inc., 80 Fifth Avenue, New York.

place, the vacuum from a good water pump is applied. The 50 cc. Pyrex centrifuge tube *B* containing the 1 to 5 cc. sample of unknown (containing 0.002 to 0.05 mg. of  $\text{NH}_3\text{-N}$ ) and 1 drop of antifoam is attached and cock *E* is opened. 2 cc. of borate buffer are placed in the side cup *C* and admitted



**Fig. 1. Distillation apparatus**

slowly through the oblique bore cock. The apparatus, suspended by the side cup from a ring-stand, is lowered into the bath by moving a single clamp and the distillation proceeds for 5 minutes. Vigorous boiling starts before the temperature inside reaches its maximum of 42.5°. Bumping is

## AMMONIA DISTILLATION

TABLE I

*Recovery of NH<sub>3</sub> on Distillation*

Corrected for distillation blank and NH<sub>3</sub> preformed in plasma. Temperature of water bath 50°.

NH <sub>3</sub> -N added mg.	NH <sub>3</sub> recovered in 5 min. from			
	5 cc. H <sub>2</sub> O		2 cc. dog plasma + 3 cc. H <sub>2</sub> O	
	mg.	per cent	mg.	per cent
0.0070	0.0069	98.6	0.0072	102.8
	0.0068	97.1	0.0071	101.4
0.0140	0.0142	101.4	0.0139	99.3
	0.0140	100.0	0.0139	99.3
0.0280	0.0285	101.8	0.0277	98.9
	0.0279	99.6	0.0277	98.9
0.0420	0.0415	98.8	0.0417	99.3
	0.0415	98.8	0.0417	99.3
Average		99.5		99.9
Total . 0.1820	0.1813	99.6	0.1809	99.4

TABLE II

*Comparison of NH<sub>3</sub> Content of Dog Urine by Distillation and Aeration Titration*

Dog	Interval No.	Vacuum distillation	Aeration
		mg. NH <sub>3</sub> -N per min.	mg. NH <sub>3</sub> -N per min.
A	1	0.562	0.568
		0.562	0.568
	2	0.605	0.620
		0.605	0.620
	3	0.634	0.608
B	1	0.596	0.608
		0.775	0.800
	2	0.775	0.800
		0.714	0.750
	3	0.726	0.750
		0.719	0.745
		0.715	0.745

TABLE III

*Decomposition of Glutamine and Urea during Vacuum Distillation at pH 10*

NH <sub>3</sub> -N distilled during	Glutamine	Urea
	per cent amide N	per cent total N
1st 5 min.	0.33 (0.15 preformed)	0.0012 (0.0008 preformed)
2nd 5 "	0.18	0.0004
3rd 5 "	0.18	0.0004
4th 5 "	0.13	0.0003

prevented by a current of air (3 to 4 cc. per 5 minutes) delivered through 15 cm. of glass capillary<sup>3</sup> of 0.05 mm. bore. This air is previously washed free of ammonia by passing through glass wool moistened with 10 N  $\text{H}_2\text{SO}_4$  in cylinder *D*.

At the end of the 5 minute distillation the stop-cock *E* is closed and the unit disconnected. The distillate is then delivered through stop-cock *E* into a 10 cc. volumetric flask, the neck of which has an internal diameter of 9 mm. The receiving unit including the stopper is rinsed down with repeated small portions of distilled water until the volume of acid distillate and rinsing in the flask is 10 cc. It is unnecessary to rinse the side arm of *A*.

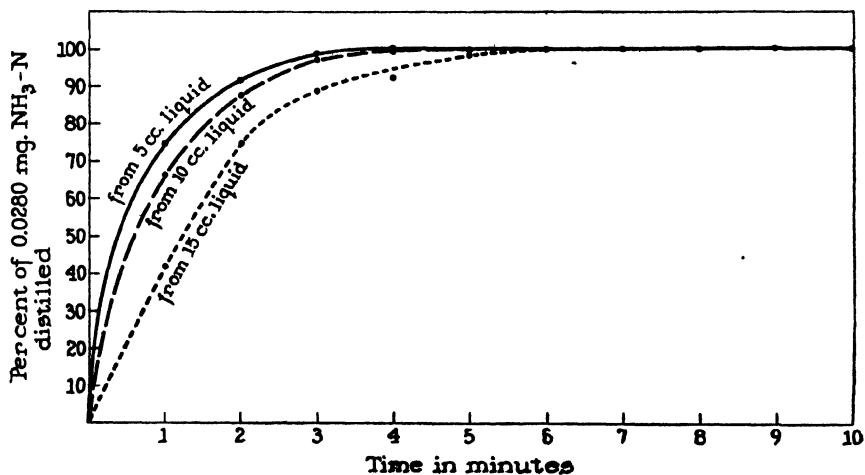


Fig. 2. Rate of distillation of  $\text{NH}_3$  at pH 10 and  $38.5^\circ$

Distillation blanks are run with, instead of an unknown, an amount of distilled water equal to that used in transferring the sample to the tube *B*.

Standards are made by adding to each of four 10 cc. volumetric flasks 5 cc. of 0.04 N  $\text{H}_2\text{SO}_4$ . Amounts of standard 0.0005 M  $(\text{NH}_4)_2\text{SO}_4$  are added to cover the desired range (e.g. 0, 1, 2, and 3 cc.) and the volume of each standard is made to 10 cc. with water.

To each standard, blank, and unknown is added 0.5 cc. of Nessler's reagent. This addition and mixing are done conveniently in the 10 cc. volumetric flasks. The absorption of light of  $\lambda = 450 \text{ m}\mu$  was measured in this laboratory in a Coleman spectrophotometer.

<sup>3</sup> Marine barometer-tubing is a sturdy capillary and is convenient to use for this purpose. It can be obtained from the Corning Glass Works, Corning, New York. Thermometer tubing with an internal diameter of 0.03 mm., though less desirable, can be used as a substitute.



### *Results*

Recoveries of  $(\text{NH}_4)_2\text{SO}_4$  on distillation from  $\text{H}_2\text{O}$  and after addition to plasma are shown in Table I.

Determinations of ammonia in diluted dog urine are shown in Table II in which the values obtained on distillation are compared with the ammonia content as measured by the Van Slyke-Cullen (7) aeration titration technique. The slightly higher values obtained by the latter technique are due partly to ammonia formed by decomposition of small amounts of glutamine in the urine.

The rate of distillation of ammonia from 5, 10, and 15 cc. of solution is indicated in Fig. 2. The rate of decomposition of glutamine and urea under these conditions is indicated in Table III. Results already obtained by this technique and which indicate the ammonia and glutamine content of dog and human plasma and urine as well as results similarly obtained in the study of conditions affecting the rate of action of glutaminase are to be reported subsequently.

### *Discussion of Method*

The usual precautions observed in micro ammonia determination should be observed. While the acidified glass wool trap removes ammonia from the air entering the unit during distillation and in the course of several hundred determinations collects considerable ammonia, the amount of air so washed is small compared with the volume of the apparatus and the air which bathes the walls of the tubes and wash bottle during transfer of specimens. It is therefore essential to work in an atmosphere as free from ammonia as possible. As was pointed out by Needham (8), tobacco smoke must be avoided.

Tributyl citrate alone is almost as effective as the antifoam mixture recommended and can be used alone if necessary. Caprylic alcohol distils away so rapidly that it loses its effectiveness as an antifoam material before the 5 minute distillation period is over. It can be used, however, if fresh portions are added during distillation and if gluconate persulfate (1) is added to the distillate to prevent the formation of turbidity on nesslerization. With colloid-free water solutions no antifoam material need be added, but with tissue extract, and especially with fresh plasmas, such a reagent is necessary. With some plasmas it is necessary to add a 2nd drop through the side arm after the 3rd minute. The tributyl citrate-methyl benzoate mixture neither gives rise to turbidity in the distillate nor decreases the intensity of the color produced with Nessler's reagent. The use of the specified antifoam material and of the trap *H* results in a minimum

mechanical transfer of spray even when bubbles of the vigorously boiling liquid in the tube *B* rise to touch the trap.<sup>4</sup>

That this method might be applicable to blood analysis, mechanical carry-over of spray and foaming have been reduced by cutting the air current to the minimum compatible with even boiling. The ammonia is transported chiefly by a current of water vapor. Distillation of 3 cc. of water provides a vapor current equivalent to 4 liters of air. The resistance to vapor flow in the bore of the stop-cock *E* and the capillary above *E* is a critical factor affecting the rate of boiling, the temperature of the contents of the tube *B*, and, when protein solutions are used, the degree of foaming. Even small departures from the specifications can appreciably affect the temperature and pressure gradients, the percentage recovery of ammonia, and the degree of breakdown of thermolabile compounds. When the temperature of the bath is 54°, the temperature in *B* at the end of the 5 minute distillation period is 40°, if 2 cc. each of standard  $(\text{NH}_4)_2\text{SO}_4$  and alkaline buffer are present. If 2 cc. each of plasma and buffer are in *B*, the maximum temperature there is 43.5°. When the bath is at 50°, the temperatures in *B* are 38.5° and 42.0° respectively. The temperatures and times outlined apply only to the apparatus specified, and it should be emphasized that the specifications given are those found best for the handling of small volumes of tissue extracts and plasma. When larger containers (*B*) are used to facilitate removal of ammonia from volumes of fluid greater than 10 to 15 cc., a standardization curve similar to those in Fig. 2 should be made for each container. The use of larger containers will increase the temperature attained therein. The extent of this increase will depend on the thickness and area of the walls. If labile amide N is present, the bath temperature should therefore be lowered and the duration of distillation increased accordingly.

The two rubber stoppers provide flexibility and facilitate rapid handling. The surface of rubber exposed in the unit is small and although transfer of ammonia to and from the rubber in these stoppers does take place to a small extent the error resulting is small. For most purposes the greater flexibility of rubber connections is to be preferred to a rigid all-glass apparatus.

<sup>4</sup> When unknowns contain a high concentration of detergents such as Aerosol, Duponol, or Turkey red oil (dioctyl sodium sulfosuccinate, sodium dodecyl sulfate, or sodium ricinoleyl sulfate), it is necessary to add to the aliquots of unknown and to the corresponding blanks 2 cc. of mineral oil or 2 to 4 cc. of kerosene, or both, and to increase the length of the distillation period by 50 per cent. When addition of kerosene is necessary, it is convenient to use 2 cc. of a light grade lubricating oil (such as S. A. E. No. 10) which already contains kerosene. The oil which distills over causes slight but constant turbidity and increases the distillation blank slightly but does not interfere with the subsequent nesslerization.

Before placing new rubber stoppers in service it is essential to boil them for an hour with normal NaOH.

As indicated by Pucher *et al.* (5), it has been found necessary to run and discard two blank distillations prior to each series of analyses in order to remove ammonium salts which accumulate on the apparatus as it stands in the laboratory.

When a series of distillations is carried out, each complete operation requires between 7 and 8 minutes. It is convenient to use alternately for *B* two centrifuge tubes, one being charged with unknown while the other is attached to the distillation unit.

The author is indebted to Dr. D. D. Van Slyke and to Dr. P. B. Hamilton for many helpful suggestions and to Dr. Hamilton for most of the glass blowing required during the evolution of the distillation unit.

#### SUMMARY

A simple accurate micro distillation technique is described suitable for use in the quantitative determination of  $\text{NH}_3$  when labile  $\text{NH}_2$ -yielding material is present.

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## AMINO ACID STUDIES

### III. THE REVERSIBILITY OF PLASMA AMINO ACID RETENTION DURING RECOVERY FROM DIETARY HYPOPROTEINEMIA IN THE DOG\*

By E. GOETTSCH, JOHN D. LYTTLE, W. M. GRIM, AND P. DUNBAR

*(From the Department of Pediatrics, College of Physicians and Surgeons,  
Columbia University, New York)*

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As hypoproteinemia developed in the dog on a low protein diet, progressive retention<sup>1</sup> of plasma  $\alpha$ -amino acids was noted after the administration of amino acids by vein or by mouth (1). The liver function of such an animal is impaired (2); at times there is morphological evidence of injury (2-4). Experimental studies indicated that the retention of plasma amino acids was probably due to reduction in deamination. When protein was restored to the diet of the hypoproteinemic dog, regeneration of serum albumin occurred rapidly (4) except after prolonged deproteinization. It was inferred that liver dysfunction might similarly be reversible under these circumstances. To test the hypothesis data for amino acid loading curves were obtained during regeneration of the tissues of the hypoproteinemic dog when protein was replaced in the diet.

#### *Procedure*

A young female dog of 13.8 kilos body weight was fed a low protein carrot diet (3) with 45 gm. of casein added daily to fulfil the normal protein requirement. A curve for plasma amino acid was obtained after the intravenous injection of casein hydrolysate,<sup>2</sup> the amount of which was 2 cc. of a 10 per cent solution per kilo of body weight diluted with an equal volume of 5 per cent glucose in isotonic sodium chloride solution. Analysis of the 10 per cent solution of casein hydrolysate, as prepared for intravenous use, revealed a pH of 5.6, an  $\alpha$ -amino acid N content of 7.39 mg. per cc., and a total N of 13.72 mg. per cc. Blood was withdrawn from the jugular vein before and after the intravenous injection; namely, 5 minutes, 15 minutes, 30 minutes, 1 hour, and 2 hours. Then the casein was removed from the diet and loading curves were obtained during deproteinization at intervals

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<sup>1</sup> Retention will be used throughout the paper to connote increased plasma values of amino acid N above the control level at any time interval following the injection of casein hydrolysate.

<sup>2</sup> An enzymatic hydrolysate of casein kindly furnished by Mead Johnson and Company.

TABLE I  
*Plasma Amino Acid N, Blood Urea N, and Hematocrit Values before and after Injections of Casein Hydrolysate in Dog 872 on Normal Diet, during Deproteinization, and during Regeneration on Normal Diet*

The plasma amino acid N and blood urea N are expressed in mg per 100 cc, the hematocrit values in per cent, and serum protein in gm. per 100 cc

Diet	Time on diet	Serum		Plasma amino acid N						Blood urea N						Hematocrit								
		Albu- min	Glob- ulin	Be- fore	After injection, min						Be- fore	After injection, min						Be- fore	After injection, min.					
					5	15	30	60	120	5		15	30	60	120	5	15		30	60	120			
Normal	6 wks.	2.80	2.92	4.96	8.74	6.49	5.19	4.27	4.96	6.03	6.09	6.54	6.37	6.92	6.58	42.3	38.4	38.6	38.8	36	8	38	1	
Low protein	1 wk.	2.59	3.07	4.62	9.48	7.65	6.01	5.26	5.01	4.96	5.32	5.61	6.06	6.42	6.36	41.5	35.6	37.5	38.0	38.2	37	7		
"	4 wks.	2.13	2.95	5.09	10.53	8.49	7.14	5.65	4.50	5.05	5.16	5.41	6.66	7.34	7.17	43.0	38.8	41.3	42.0	38.2	39.9			
"	9 "	1.52	2.23	3.34	9.36	6.53	4.81	3.70	3.34	1.98	2.10	2.96	3.23	2.70	2.56	35.6	33.9	35.8	35.1	32.5	33.5			
Normal	2 days	1.42	1.95	6.93	11.37	8.94	7.87	7.69	6.96	4.30	3.71	4.62	4.74	3.95	3.28	52.8	48.3	52.8	52.8	72.8	72.7	52.7	0	
"	7 "	1.89	2.33	6.29	10.40	8.48	6.28	5.74	5.35	5.07	5.81	6.10	6.26	7.30	6.99	28.7	28.8	28.9	28.9	27.7	26.4			
"	16 "	2.36	2.63	5.39	9.29	7.41	5.71	4.96	4.77	6.72	6.16	6.49	6.62	6.95	7.20	32.4	30.1	30.7	30.5	29.5	29.5			

of 1, 4, and 9 weeks after the low protein diet was begun. Casein was restored to the diet and similar studies were carried out on the 2nd, 7th, and 16th day of regeneration. Plasma amino acid N was determined by the gasometric ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (5); blood urea by the urease method of Van Slyke (6). Other analytical methods and technical details have been described previously (1).

### *Results*

As hypoproteinemia developed, progressive retention of plasma amino acid was noted. The data (Table I) for the initial amino acid loading curve for a normal dog on a complete diet revealed the characteristic pattern observed previously (1), a prompt rise and fall in the concentration of plasma amino acid N within 30 minutes, a drop below the preinjection value, and subsequent return to the initial level at the end of 2 hours. The injected amino acids were all cleared from the plasma 30 minutes after the injection. After 1 week on the low protein diet the concentration of plasma amino acid N was higher than in the normal curve at all intervals except the last at 2 hours, and retention of plasma amino acid was further increased after 4 and 9 weeks on the deficient diet.

With regeneration of the serum albumin and tissues after casein was restored to the diet retention of plasma amino acid receded quickly. Within 2 days marked acceleration of the disappearance of amino acids from the plasma was observed and the curve at this time resembled that obtained after 1 week of deproteinization in so far as the increase above the preinjection value was concerned. On the 7th day of regeneration further improvement had occurred and on the 16th day of regeneration the loading curve obtained closely resembled the original curve on the normal dog before deproteinization.

In agreement with previous results changes in blood urea N and hematocrit during the individual amino acid loading test were slight. During deproteinization the concentration of blood urea fell slowly and returned to the normal value rapidly on restoration of casein to the diet. The maintenance of the total red blood cell volume at the normal level during the first 4 weeks of deproteinization and subsequent fall have been described (7). Failure of the hematocrit readings to return to the normal level on addition of casein to the diet in the present series of observations probably represents insufficient allowance of time for recovery of this feature. The decline in concentration of serum albumin and in hematocrit levels after 2 days of casein refeeding undoubtedly represents changes due to increase in plasma volume rather than a loss of total circulating albumin (7). The fasting levels of plasma amino acid N obtained during regeneration were higher than those obtained in the normal dog.

*Comment*

It is apparent from the studies reported here in the dog that the retention of plasma amino acids found in the hypoproteinemic state is reversible on replacement of protein in the diet. The plasma is cleared of injected amino acids at the normal rate before the serum albumin is completely regenerated and before any restoration of red blood cell volume has taken place. Since in the hypoproteinemic dog the retention of plasma amino acid is closely correlated with liver injury (1), it seems logical to deduce that recession of the retention of plasma amino acid indicates reversible disturbance of liver function.

## SUMMARY

As hypoalbuminemia developed in the dog, amino acid loading tests revealed progressive failure to clear the plasma of  $\alpha$ -amino acid N. The changes were apparent within 7 days on the low protein diet.

On restoration of protein to the diet with regeneration of serum albumin the lag in plasma clearance disappeared equally rapidly.

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# COMPARISON OF THIAMINE VALUES BY CHEMICAL AND BIOASSAY METHODS

By ELMER B. BROWN, JOHN C. HAMM, AND HORACE E. HARRISON

(From the Anheuser-Busch Laboratories, St. Louis, Missouri, and  
Old Bridge, New Jersey)

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The determination of thiamine in widely different products has become a necessity as a result of the enrichment program, providing for a minimum and maximum vitamin content. However, the development of a completely suitable and accurate method for the determination of thiamine has proved a difficult task. The chemical method of Hennessy and Cerecedo (1) has proved the most applicable of the various methods proposed for the determination of thiamine and is the method most commonly employed for this purpose. Since its publication a number of modifications have been proposed, varying the reagents and technique to be applied for optimum results, and studies have been made by various groups to determine its applicability to different types of products. Collaborative<sup>1</sup> tests by a large number of chemists over an extended period of time show appreciable variation in results among the collaborators on the same samples and that their average values are lower than bioassay values.

We participated in some of these collaborative tests and in addition carefully analyzed the samples for their bioassay values. A comparison of the results obtained and the specific procedure we use for thiamine analysis we believe will prove useful. Usually the modifications we have made in the Hennessy and Cerecedo procedure are slight and were made after extensive study of each step of the process. Only such modifications were adopted as tend to give results more in line with bioassay values. Even with these modifications, our results are about 10 per cent below the bioassay values, whereas the average collaborative results, obtained by other modifications of the method, are 20 per cent below the bioassay values.

## EXPERIMENTAL

*Activation of Decalco and Preparation of Adsorption Tubes*—The Decalco (60-80 mesh) is activated by being washed four times for 15 minutes each with four volume portions of hot 5 per cent acetic acid solution; it is then washed four times with four volume portions of 25 per cent KCl solution,

<sup>1</sup> Unpublished results from groups of collaborators studying the thiochrome method on various samples submitted by different laboratories.



and finally twice with distilled water. It is important that all of the wash solutions be kept hot during the wash period and agitated constantly. The activated Decalso should not be dried but stored under distilled water.

A ball of cotton 2 cm. high is placed in the bottom of the adsorption tube over the capillary and the tube filled with distilled water. The wet Decalso is added, a portion at a time, and allowed to settle by gravity to fill the tube to a height of  $3\frac{1}{2}$  to 4 inches. The height of the Decalso should be the same in all tubes. During the passage of the sample and the subsequent washings, the Decalso will settle somewhat. The water is allowed to drip through by gravity, followed by 5 ml. of acidified KCl solution (250 gm. of KCl plus 10 ml. of concentrated HCl per liter), then by 50 ml. of hot distilled water. The tubes may be stored in a beaker, with distilled water high enough to cover the Decalso in the tubes, for a few hours until the samples for adsorption are ready. Immediately before use, 5 ml. of 2 per cent acetic acid solution are passed through the tube, followed by 5 ml. of distilled water and then the sample. Only one sample should be put through the adsorption tube without reactivation of the Decalso. The Decalso can be recovered from the tubes and accumulated for reactivation if desired. Not more than three reactivations of the Decalso should be made before the Decalso is discarded.

*Preparation of Sample*—The sample must contain approximately the same amount of thiamine as the standard (0.2  $\gamma$ ), so that the deflections between the two will be ideally comparable. The wider the range the less accurate is the relationship between the standard and the unknown. We use a 5 ml. stock solution of standard (U.S.P. thiamine hydrochloride dried over  $P_2O_5$ ) containing 100  $\gamma$  per ml. or 500  $\gamma$  of thiamine for subsequent refluxing, diastatic incubation, and filtration. The standard and unknown are treated identically, with the various dilutions made at the same point.

5 ml. of standard are made up to 200 ml.; 10 ml. of this dilution are made up to 100 ml. and filtered. 20 ml. of this solution are passed through the column and washed with 45 ml. of hot distilled water. After the columns have been washed, the sample is eluted with 25 ml. of acidified KCl solution. 5  $\gamma$  of standard are now contained in 25 ml. of KCl eluate or 1  $\gamma$  per 5 ml. The concentration of the thiamine in the unknown should be in this range.

A sample of 1 or more gm. containing a minimum of 10  $\gamma$  of thiamine should be used for hydrolysis. If dried enriched bread is used, a 2.0 gm. sample is suspended in 50 ml. of 0.1 N  $H_2SO_4$  and heated with occasional swirling at the boiling temperature under a reflux on an asbestos-covered hot-plate for 30 minutes after the sample has reached the boiling point. The flask is then removed and cooled to approximately 55°. 20 ml. of taka-diastase solution are added and the suspension incubated at 50–55° for 2 hours. The taka-diastase solution is made by dissolving 1.25 gm. of taka-diastase in 100 ml. of buffer solution. The buffer solution is made

in liter lots by dissolving 136 gm. of sodium acetate and 60 ml. of glacial acetic acid in distilled water. The 20 ml. of diastase solution contain 0.25 gm. of taka-diastase; it buffers the suspension to pH 4.5 to 4.7. The taka-diastase solution should be made up daily. After incubation, the enzymatic activity is checked by the addition of 5 ml. of  $N H_2SO_4$ . The sample is now diluted with distilled water to 100 ml. and filtered through No. 1 Whatman filter paper. A sufficient amount of the filtrate is passed through the prepared column so that the final 25 ml. of KCl eluate contain approximately 5  $\gamma$  of thiamine, as does the standard. The standard sample is diluted and filtered at this point, as is the unknown.

When the samples have passed through the column, each column is washed with two 10 ml. portions of hot distilled water and then 25 ml. of hot distilled water. When the last wash water has reached the top of the Decalso, 25 ml. of acidified KCl solution are passed through the column. All of the KCl solution is allowed to drip through and collect in a 25 ml. glass-stoppered graduated cylinder. If the cylinder is not filled exactly to the 25 ml. mark, it is adjusted to the mark with a few drops of acidified KCl.

*Preparation of Sample for Fluorometric Reading*—5 ml. of standard eluate containing 1  $\gamma$  of thiamine are pipetted into each of two centrifuge tubes. To one are added 3 ml. of 15 per cent NaOH and 25 ml. of redistilled isobutyl alcohol in the order named. To the other are added 3 ml. of 15 per cent NaOH, 0.2 ml. of 1 per cent potassium ferricyanide solution, and then after 30 seconds 25 ml. of redistilled isobutyl alcohol. The two tubes are stoppered and shaken for 90 seconds and centrifuged briefly. 20 ml. of the alcohol layer of each tube are pipetted into dry centrifuge tubes containing 1 to 1.5 gm. of anhydrous sodium sulfate, shaken, and centrifuged briefly. The dried alcohol solutions are poured into the fluorophotometric cuvettes and are ready to be read. The unknown sample is treated in the same manner and at the same time intervals checked by the stopwatch.

*Standardization of Fluorophotometer and Sample Readings*—We use a secondary standard of quinine sulfate (0.27  $\gamma$  per ml. made up in 0.1  $N H_2SO_4$ ) solution which we set by means of the iris diaphragm to give an  $F$  reading of 60. Then without changing the iris, we adjust the resistance switch to give a  $T$  reading of 90. This quinine standard is used before each assay reading in order to determine whether the light source has remained constant. If it has changed, the  $F$  reading is readjusted by means of the iris back to 60 and the  $T$  reading to 90 by means of the resistance switch. At the concentration of the above quinine sulfate and settings for  $F$  and  $T$ , 1  $\gamma$  of thiamine hydrochloride will give an  $F$  reading of 30 to 40 deflections, depending upon light intensity and other factors.

With the fluorophotometer standardized as described, the quinine sulfate

is removed and the oxidized sample is read. Both  $F$  and  $T$  readings are made. The transmission reading is then divided into the fluorescent reading to give the corrected fluorescent reading on the basis of 100. This gives a standard transmission reference point that corrects the difference in color developed in the extract. The difference between the corrected oxidized  $F$  reading and the corrected unoxidized  $F$  reading represents the thiochrome produced and this difference gives deflections per microgram of thiamine hydrochloride, since 1  $\gamma$  was measured.

*Example—*

Standard unoxidized.	$F = 5.5$	$\frac{5.5}{90} = 6.11$
“ “	$T = 90.0$	
“ oxidized.	$F = 36.0$	$\frac{36}{91.5} = 39.34$
“ “	$T = 91.5$	

Hence  $39.34 - 6.11 = 33.23$  deflections per microgram.

*Notes*—The isobutyl alcohol is recovered by distilling in a glass still equipped with a centrifgrade thermometer. Only that portion of alcohol that distills above  $103^\circ$  is collected.

Corks may be used in place of glass stoppers, provided they are covered with aluminum foil.

A water bath is preferable for enzymatic incubation when the temperature is accurately controlled.

In Table I data are given on different products as determined by us, the average value of various collaborators on the same products, and the bioassay values by the rat growth method. Each of the bioassay values was determined at three levels and the final value checked.

*Behavior of Enriched Tablets*—Enriched tablets from three different companies were obtained and the thiamine content determined by the rat growth method and fluorometrically. Each tablet showed the potency claimed for it by the manufacturer on bioassay but the results obtained varied appreciably from each other by the chemical procedure and were lower than bioassay values. This difference in behavior is attributed to the difference in tablet manufacture, since it is assumed the different companies use different fillers and binders in their tablet manufacture. In Table II it will be observed that one of the tablets gives 12 per cent more thiamine when the extraction is made with KCl solution (Tablet 3, b) than when made by our regular method (Tablet 3, a). This would indicate that incomplete extraction is encountered in this particular tablet and to an extent greater than in Tablets 1 and 2, in which no such difference was found.

When, instead of pipetting an aliquot, all of the extract of Tablet 3, a was filtered and the residue thoroughly washed, we obtained a recovery of

90.6 per cent of the bioassay value. This further supports the conclusion that insoluble material left in the hydrolyzed sample may occlude some of the thiamine and prevent a homogeneous solution from being obtained in the extraction. This does not explain, however, the approximately 10 per cent higher results that the bioassay method shows over the fluorometric

TABLE I  
*Thiamine Determinations*

Material	Bioassay value	Our results			Collaborators' results	
	$\gamma$ per gm.	$\gamma$ per gm.	per cent bioassay	$\gamma$ per gm	per cent bioassay	
Fortified flour. . .	7.0	6.26	90.3	5.37	76.7	
Enriched, phosphated flour	5.3	4.77	89.4	4.03	80.6	
Wheat germ	32.0	28.30	90.0	26.50	83.7	
Enriched bread	3.2	2.55	80.0	2.59	80.9	
“ “	4.4	3.98	90.3	3.30	76.9	
“ “	4.55	4.30	94.5	3.46	76.2	
“ “	4.45	3.85	86.5	3.52	79.1	
“ “	5.47	5.11	93.4	4.71	86.1	
“ flour	4.76	4.59	96.3	3.89	81.7	
Dried yeast	17.90	15.20	85.0	12.80	71.5	
Average % of bioassay			89.6		79.3	

\* The bioassay values were determined by H. M. Baum and F. M. Younger of the Biological Laboratories of Anheuser-Busch, Inc. The authors wish to acknowledge their valuable assistance.

TABLE II  
*Results of Bioassay and Thiochrome Methods on Enriched Tablets*

Tablet No (bioassay value 150 mg)	Fluorometer reading	Per cent of bioassay value
	mg	
1	131.06	87.3
2	133.20	88.8
3, a	119.50	79.6
3, b	136.90	91.3

method. Since this percentage varies with different products, it is assumed that some deterioration of the thiamine has taken place that does not prevent utilization by the rat but that is not measured by the chemical method.

*Thiamine Values of Dough and Bread by Bioassay and Fluorometric Determinations*—Two doughs were prepared of which one contained only regular ingredients, while the other was enriched. Bioassay was carried

out on the doughs and the bread and the results compared with those obtained by chemical determination. In the chemical determination, the entire sample hydrolyzed was thoroughly washed and the residue tested to make sure that no thiamine was occluded by the residue. These data are given in Table III.

When aliquots of these sample solutions were pipetted off and analyzed, the values obtained were lower, being as low as 80 per cent of the bioassay values in some instances. It is interesting to note that the baking loss of thiamine between the ingredients and the bread was the same whether calculated from the bioassay results or from the fluorometric values, and that the percentage of thiamine loss in the regular bread was appreciably higher (20.5 per cent) than in the enriched bread (15.5 per cent). A similar result in the loss of thiamine in toasting regular and enriched bread was reported by Downs and Meckel (2) who used the fermentation method for their assays; they stated, "The apparent percentage loss of thiamin in the

TABLE III  
*Results of Bioassay and Fluorometric Determinations on Dough and Bread*

	Bioassay method	Fluorometric method	Per cent of bioassay value
	$\gamma$	$\gamma$	
Regular ingredients	368	334	90.8
" bread	291	264	90.9
Enriched ingredients	1718	1562	90.5
" bread	1446	1314	90.2

toast made from unenriched white bread is higher than thiamin losses from either the enriched white bread or the 100% whole wheat bread."

#### DISCUSSION

In the preparation of the sample we find that the acid hydrolysis described is sufficient and better suited for the subsequent enzymatic treatment than is the more drastic hydrolysis under pressure, as recommended by some operators. This step is not as important as the enzymatic digestion which we carry out with taka-diastrase at 52–55°, a temperature higher than is recommended in other procedures. Our experiments show that in most cases complete hydrolysis and destruction of the cozymase is accomplished with taka-diastrase in a period of 2 hours at this temperature and further treatment overnight does not increase the thiamine content. A sample of bread when digested for 2 hours and overnight gave 3.22 and 3.27  $\gamma$  per gm. respectively.

Taka-diastrase has proved more suitable for the enzymatic digestion than

either clarase or polydase. We have been unable to obtain the higher results by the use of polydase than by taka-diasatase reported by Clausen and Brown (3). In fact, our results were more erratic when polydase was used than with taka-diasatase. Our analyses show that the polydase samples we obtained were not uniform and contained appreciable amounts of thiamine that increased the sample values from that source instead of by the liberation of bound thiamine.

The preparation and activation of Decalso has proved a difficult task. Some preparations of Decalso we have obtained could not be activated sufficiently to adsorb all of the thiamine from the extract. Other samples had a tendency to oxidize the thiamine to an appreciable extent in passing through the column, introducing an error in the extraction. Drying the activated Decalso proved to be detrimental to its adsorption properties; also the dried Decalso gradually lost its effectiveness in adsorption on standing. Within a period of 30 days, one batch of dried, activated Decalso would give only 50 per cent of the thiamine value originally obtained on the same sample used daily as a check. Reactivation of the Decalso and bioassay showed that the deterioration was in the Decalso and not in the sample. Storage under distilled water has proved most satisfactory after the Decalso has been activated. Decalso containing a large percentage of iron has been found to be poorer in adsorption properties than purer products. The iron from this material can be removed by treatment with HCl, but this treatment in some instances did not produce a good quality of Decalso, while in others it did.

The calculation of the fluorometric readings to a standard transmission reference point eliminates a source of error due to color developed in the extracts as compared to the less colored standard. This calculation is based on the assumption that any color that affects the intensity of the transmission rays likewise affects the fluorescent readings. The corrected readings, in the case of colored solutions, agree more nearly with the bioassay results than the uncorrected readings, and hence have been adopted as standard in our laboratories.

The presence of fluorescent material in some samples of anhydrous sodium sulfate has been observed. Since sodium sulfate is used to remove the water from the isobutyl alcohol solutions, an excess only is required and the amount used is not accurately weighed. If it contains fluorescent materials, erratic results will be produced. In the samples of sodium sulfate in which fluorescent material was found, the fluorescence was destroyed by heating in the muffle furnace at ashing temperature.

We do not find that the method is sufficiently flexible to permit the time intervals recommended by the United States Pharmacopoeia Vitamin Committee (4) for thiamine determination. Only by use of a stop-watch

and definite time intervals of the magnitude described in our procedure are we able to obtain maximum results. When the time intervals recommended by the Vitamin Committee are used, the results obtained on aliquots of the same sample are only 70 to 80 per cent of the maximum values obtained by the method under optimum conditions.

With compulsory enrichment of food materials, the accurate determination of thiamine in a number of products has become of commercial importance to the producers of food products. If the available methods for thiamine determination show as a maximum only 80 to 90 per cent of the thiamine value of these materials, the question arises as to how this should be interpreted at the present time. The amounts of thiamine required are based on bioassays, unsuitable for commercial control, but give values from 10 to 20 per cent higher than the chemical methods that are suitable for commercial control. The value of 10 to 20 per cent of the thiamine used for this purpose amounts to millions of dollars annually, an amount sufficiently large to attract the attention of users of this material for fortification purposes.

#### SUMMARY

A study of the thiochrome method for the determination of thiamine has been made; thiamine values determined by this method are only 80 to 90 per cent of the bioassay values.

Insoluble material produced in the hydrolysis of the sample may occlude thiamine to such an extent as to become a chief source of error when aliquots of the hydrolyzed samples are used. Thorough washing of the residues to remove occluded thiamine before aliquots are taken gives higher results for thiamine but the time and work required for this step are objectionable factors.

The basic method of Hennessy and Cerecedo is used and minor modifications are made that tend to bring the results more in line with bioassay values. These modifications include definite optimum time intervals for different steps in the process and a higher temperature (52–55°) for the enzymatic hydrolysis with taka-diaxase. Clarase and polydase preparations were not as suitable as taka-diaxase for the enzymatic hydrolysis under the conditions employed (as taka-diaxase).

The preparation of the Decalso is an important part of the process and requires careful attention to maintain satisfactory activated Decalso for maximum adsorption and elution. Drying the activated Decalso was found objectionable and the activity of dried Decalso unstable.

The calculation of the readings to a standard transmission reference point corrects the difference in readings due to differences in color between the extracts of sample and standard. This calculation of readings gives

values that are closer to the bioassay results. The sample extracts contain more color than the standard and this color affects the reading of the thiochrome to a greater extent in the sample than in the standard.

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# THE INFLUENCE OF SUGARS ON THE FORMATION OF SULFHYDRYL GROUPS IN HEAT DENATURATION AND HEAT COAGULATION OF EGG ALBUMIN

By CHARLES D. BALL, CHESTER R. HARDT, AND WILLARD J. DUDDLES

*(From the Kedzie Chemical Laboratory, Michigan State College of Agriculture and Applied Science, East Lansing)*

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Beilinson (1) in 1929 showed that sucrose and glycerol inhibited the heat coagulation of egg albumin. Bancroft and Rutzler (2) claimed that the sugar peptized the coagulated protein. Harris (3) by the use of the nitroprusside reaction showed that sulfhydryl groups were not present in raw egg white but their presence could be demonstrated after heat coagulation. Various workers (4-7) have reported the formation of compounds between sugars and amino acids, simple peptides, and, in some cases, between sugars and proteins. Schubert (8) prepared crystalline compounds of a number of sugars with cysteine, compounds in which the nitroprusside test was negative. Von Przylecki and Cichocka (9) formed compounds which they called "symplexes" with maltose and several proteins. The "symplexes," formed at pH values between 7 and 10, were believed to involve the lysine residues of the proteins. No such compound was formed with sucrose.

In the present work, the protective action of sugars toward coagulation was thought to be due to the influence on the sulfhydryl groups in the denaturation phase of the process. To obtain evidence on this point, egg albumin solutions were heated in the presence and in the absence of sugars, and the sulfhydryl content of the egg albumin was determined by two different methods. The inhibition of heat coagulation exerted by sugars has also been shown by determining the amount of uncoagulated nitrogen when samples of egg albumin were heated in the presence and absence of sugars.

To indicate whether "symplexes," similar to those described by von Przylecki and Cichocka, were involved in this protective action, samples of egg albumin were allowed to stand at 5° for 96 hours at pH 8.6 and also at pH 4.8. After adjustment to the same pH, the amount of uncoagulated nitrogen was determined in each case. A coagulated "symplex," prepared similarly to that described by the above workers, has also been prepared and the amount of reducing substances present in this "symplex" compared with that present in ordinary heat-coagulated egg albumin.

## EXPERIMENTAL

The experimental work was divided into three parts: (1) the influence of sugars and mannitol on the liberation of sulfhydryl groups; (2) the influ-

ence of sugars on the heat coagulation of egg albumin; (3) an attempt to show protein-carbohydrate combination.

Egg albumin was prepared by the method of Kekwick and Cannan (10). Dialysis of the sodium sulfate was carried out under reduced pressure. The nitrogen content of the egg albumin solution was determined by a micro-Kjeldahl method (11).

Two methods were used to determine the sulfhydryl groups, neither of which involved the probability of denaturation of the protein prior to the determination. The first of these methods involved the use of iodoacetic acid as described by Rosner (12); the second utilized the phenolindo-2,6-dichlorophenol titration as described by Todrick and Walker (13).

For the iodoacetic acid method, standard solutions of recrystallized cysteine hydrochloride containing the equivalent of 0.07 to 0.35 mg. of cysteine per ml. were prepared. To 1.5 ml. portions of these solutions were added 4.5 ml. of distilled water. The mixture was heated at 70° for 15 minutes and then allowed to cool. 2 ml. of a phosphate buffer, pH 7.4, and 2 ml. of 0.1 N iodoacetic acid, previously neutralized to pH 7.4, were added, and the mixture was allowed to stand for 30 minutes. At the end of that time, 0.25 ml. of a solution of 10 per cent trichloroacetic acid by weight and 0.25 ml. of a solution of 10 per cent sulfuric acid by weight were added and allowed to stand for 5 minutes and the solution was then filtered. To the filtrate was added 0.25 ml. of 3 per cent hydrogen peroxide by weight and the mixture was made up to 10 ml. with distilled water. After it had stood for 35 minutes, the color developed was determined by use of a photometer with a blue filter having a maximum absorption range of 4500 to 5000 Å. The standardization repeated with solutions of *d*-glucose in place of distilled water gave identical results with those obtained without glucose. The egg albumin solution was adjusted to pH 4.8 with 0.1 N hydrochloric acid. 1.5 ml. aliquots, containing 40.62 mg. of egg albumin per ml., were pipetted into test-tubes, and 4.5 ml. of the sugar solution or distilled water added. These mixtures were then treated in exactly the same manner as described in the standardization procedure. The mg. of cysteine were read from the standardization curve. The results with *d*-glucose, *d*-fructose, *d*-mannose, *l*-arabinose, *d*-xylose, and the hexatomic alcohol *d*-mannitol are given in Table I. Mannitol was added to this group of sugars to compare the effect of the absence of a carbonyl group. Each figure given in Table I represents the average of from seven to ten determinations. Results in the table have been corrected for blanks on the reagents. The addition of unheated egg albumin to these blanks did not in any case change their value.

For the titration method with phenolindo-2,6-dichlorophenol, cysteine hydrochloride was also used for standardization. A weighed amount of the recrystallized cysteine hydrochloride was first neutralized in an atmosphere

of nitrogen, dissolved in 2 ml. of a phosphate buffer, pH 4.8, and made up to 10 ml. with freshly boiled distilled water. The solution was then titrated in an atmosphere of nitrogen with a 0.1 per cent solution of phenolindo-2,6-dichlorophenol. 0.1 mg. of cysteine was found to be equivalent to 3 ml. of the phenolindo-2,6-dichlorophenol. 1.5 ml. aliquots of an egg albumin solution, containing 40.62 mg. of egg albumin per ml., were then pipetted into test-tubes. To each of these were added 4.5 ml. of distilled water, or sugar solution, 2 ml. of buffer, pH 4.8, and 2 ml. of distilled water.

TABLE I  
*Influence of Inhibiting Substances on Apparent Cysteine Content of Heat-Denatured Egg Albumin*

Inhibiting substance	Concentration	Cysteine in 66.93 mg egg albumin			
		Iodoacetic acid method		Phenolindo-2,6-dichlorophenol method	
	<i>M</i>	<i>mg.</i>	<i>per cent</i>	<i>mg.</i>	<i>per cent</i>
Water		0.359	0.589	0.343	0.563
<i>d</i> -Glucose	0.045	0.342	0.562	0.329	0.540
	0.225	0.289	0.475	0.282	0.463
	0.450	0.220	0.361	0.215	0.353
<i>d</i> -Fructose	0.045	0.347	0.570	0.314	0.516
	0.225	0.296	0.486	0.298	0.490
	0.450	0.251	0.412	0.260	0.427
<i>d</i> -Mannose	0.045	0.353	0.580	0.336	0.551
	0.225	0.310	0.510	0.302	0.496
	0.450	0.258	0.423	0.256	0.420
<i>l</i> -Arabinose	0.045	0.354	0.581		
	0.225	0.301	0.494		
	0.450	0.250	0.410		
<i>d</i> -Xylose	0.045	0.359	0.589		
	0.225	0.309	0.507		
	0.450	0.265	0.436		
<i>d</i> -Mannitol	0.045	0.363	0.600		
	0.225	0.355	0.583		
	0.450	0.293	0.481		

Varying amounts of phenolindo-2,6-dichlorophenol were then added and the tubes heated at 70° for 15 minutes. The tubes that were decolorized within that time were noted, and a further series of tubes made up, containing quantities of reagent distributed over a narrower range. The accuracy in this work appeared to be about 0.5 ml. of the phenolindo-2,6-dichlorophenol, although the originators of the method claimed to be able to distinguish differences to 0.01 ml. The results with this method are presented in Table I.

The influence of sugars and *d*-mannitol on the inhibition to heat coagula-

tion was studied in the following manner. 1.5 ml. of an egg albumin solution containing 40.62 mg. of egg albumin per ml. were pipetted into test-tubes and 4 ml. of a phosphate buffer, pH 4.8, and 4.5 ml. of water, or of a solution containing the inhibiting agent, were added. The solutions were then heated at 70° for 15 minutes, filtered, and the nitrogen in a 2 cc. aliquot of the filtrate determined. The results with various sugars and mannitol are given in Table II. Each value given represents the average of at least quadruplicate coagulations and subsequent nitrogen determinations. In two additional series of experiments, sufficient *d*-glucose or *d*-fructose was added to saturate the solution of the egg albumin completely.

TABLE II

*Influence of Inhibiting Substances on Heat Coagulation of Egg Albumin*

The values given are increases in percentages, over controls, of non-coagulable nitrogen in the filtrate after removal of the coagulated protein.

Substance	Concentration of inhibiting substance			
	0.045 M	0.225 M	0.450 M	Saturated solution
<i>d</i> -Glucose	3.2	12.7	21.2	80.8*
<i>d</i> -Fructose	2.1	8.6	12.3	84.1†
<i>d</i> -Mannose	2.2	6.7	11.2	
<i>l</i> -Arabinose	7.7	20.3	32.6	
<i>d</i> -Xylose	2.2	13.7	28.4	
Sucrose	2.4	8.9	12.7	
<i>d</i> -Mannitol		9.0		16.0‡

\* About 4.6 M in respect to glucose

† About 5.0 M in respect to fructose

‡ About 0.8 M in respect to *d*-mannitol

After the period of heating as described above, the solutions were dialyzed until no reducing sugar could be detected in the diffusate with Benedict's reagent. When the egg albumin so dialyzed was adjusted to pH 4.8, no coagulation occurred.

The first attempt to determine whether "symplexes," of a type similar to those described by von Przylecki and Cichocka, might be responsible for the inhibiting influence was made by comparing the amount of coagulation of the egg albumin first treated with sugar at pH 4.8, and subsequently heat-coagulated at that pH, with that first treated with sugar at a pH of 8.6, and subsequently heat-coagulated at a pH of 4.8. The sugars were added to the egg albumin at the pH indicated and the samples were coagulated at once or else allowed to stand for 96 hours at a temperature of about 5°. At the end of that period, these samples were also coagulated and the nitrogen determined in aliquot portions of each of the filtrates. Results are presented in Table III.

The second attempt to show "symplex" formation consisted of the coagulation of 5 ml. portions of a solution containing 60.5 mg. of egg albumin per ml. in the presence and in the absence of *d*-glucose. The coagulum in each case, after being washed, was partially hydrolyzed by being heated for 8 hours with 2 per cent hydrochloric acid in an oil bath at 120° and the hydrolysate was analyzed for reducing substances. Any unhydrolyzed protein was removed with lead acetate, the excess lead removed with potassium oxalate, and the reducing substances determined by the modified Benedict method for blood sugar (14). A photometer with a green filter of maximum absorption range of 5200 to 5800 Å. was used for comparison of the amounts of color developed. The standardization curve was made with *d*-glucose solutions containing 0.05 to 0.5 mg. per ml. The average of twelve determinations of the reducing substances, calculated as *d*-glucose in the partially hydrolyzed egg albumin, was 1.26 mg. with an average

TABLE III

*Influence of Time of Contact, and pH, of Sugars on Coagulation of Egg Albumin*

The values given are for the percentage of nitrogen in the filtrate after coagulation based on the total nitrogen in the filtrate before filtration.

Sugar, 0.3 M	Coagulated at once		Coagulated after 96 hrs. at 5°	
	pH 4.8	pH 8.6	pH 4.8	pH 8.6
<i>d</i> -Glucose	12.41	58.32	11.83	58.59
<i>d</i> -Fructose	12.53	46.98	10.76	46.83

deviation from the mean of  $\pm 0.056$ , while the corresponding figure for the partially hydrolyzed egg albumin, coagulated in the presence of glucose, was 1.30 mg. with an average deviation from the mean of  $\pm 0.072$ .

#### DISCUSSION

The inhibition of sulfhydryl formation, as determined by two different methods, depends to some extent on the nature of the sugar used. The alcohol *d*-mannitol is less effective than the simple sugars. The amount of cysteine, as calculated from the sulfhydryl content, by the iodoacetic acid method, was 0.589 per cent; that by the phenolindo-2,6-dichlorophenol was 0.563 per cent. These values compare favorably with 0.56 to 0.61 per cent reported by Mirsky and Anson (15), 0.55 per cent reported by Rosner, 0.58 per cent reported by Kuhn and Desnuelle (16), 0.50 per cent reported by Greenstein (17), and that of 0.63 per cent given by Todrick and Walker (13). Based on the value 0.59 per cent, the percentage protection given by each of the different substances in Table I when used in 0.225 M concentration would be 17 per cent for *d*-glucose, 16 per cent for *d*-fructose, 14 per cent for *d*-mannose, 16 per cent for *l*-arabinose, 14 per cent for *d*-xylose, and

1 per cent for *d*-mannitol. These values are in most cases somewhat higher than the values given in Table II for the influence of the same substances on heat coagulation. The chief inference would be that some factor in addition to the effect on the sulfhydryl group is involved in the subsequent coagulation. Sørensen and Sørensen (18) a number of years ago showed that denaturation did not produce an increase in soluble nitrogen, but subsequent heating with water caused an increase in ammonia N and total soluble N.

That the inhibition is due to an influence on the native protein, and not to a peptization of the coagulated protein, is indicated both by (1) the influence on the sulfhydryl groups, and (2) the stabilization of egg albumin when saturated with *d*-glucose or *d*-fructose. In the latter cases, no coagulation occurred when practically all of the sugar was removed by dialysis, and when the pH of the solution was adjusted to the isoelectric point of the egg albumin. If the egg albumin had been previously denatured, coagulation should have occurred (19).

The failure to show more inhibiting influence at higher pH does not indicate the formation of "symplexes" as described by von Przylecki and Cichocka, neither does the observation that, coagulated in the presence of glucose, egg albumin has practically the same content of readily hydrolyzable reducing substances as of that coagulated in the absence of glucose. These "symplexes," as pointed out by von Przylecki and Cichocka, would be very labile at a pH comparable to that occurring in a cell.

Work to be reported later will demonstrate that sugars decrease the mobility of egg albumin. Preliminary work in connection with the Central Brucella Station, Michigan State College (20), has shown that bovine plasma saturated with glucose shows, after heating, a normal electrophoretic pattern.

#### SUMMARY

*d*-Glucose, *d*-fructose, *d*-mannose, *l*-arabinose, *d*-xylose, and *d*-mannitol inhibited the formation of sulfhydryl groups when egg albumin was heat-denatured under specified conditions. These same substances, as well as sucrose, increased the amount of non-coagulable nitrogen when egg albumin was heat-coagulated under similar conditions. The inhibiting influence toward heat coagulation does not increase with the increase of time of contact of the agent with the egg albumin, even at a high pH. Egg albumin coagulated in the presence of glucose does not contain significantly more readily hydrolyzable reducing substances than does egg albumin coagulated in the absence of glucose.

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# THE *d*-AMINO ACID OXIDASE, URICASE, AND CHOLINE OXIDASE IN NORMAL RAT LIVER AND IN NUCLEI OF NORMAL RAT LIVER CELLS

By TIEN HO LAN

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

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Although many enzyme systems in animal liver have been recently investigated by different workers, relatively little work has been done on the enzyme systems in isolated nuclei. Boell, Chambers, Glancy, and Stern (1) have demonstrated the cytochrome oxidase activity of nuclei prepared from egg cells of *Arbacia punctulata* by the method of Harvey (2) and Harvey (3). Behrens and his associates (4) succeeded in preparing nuclei from hepatic cells and were able to demonstrate the existence of arginase and a very small amount of lipase in their preparations. Recently Dounce (5) used a new method to prepare nuclei of rat liver in such a way that the enzymes and proteins are probably not damaged, and several enzymes were reported to be present in these nuclei. In this paper three more enzymes have been studied in nuclei of rat liver cells, prepared by the method of Dounce (5), and the concentrations of these enzymes in isolated nuclei have been compared with the corresponding concentrations in whole liver tissue. The enzymes in question are *d*-amino acid oxidase, uricase, and choline oxidase.

## EXPERIMENTAL

In this work, both Osborne-Mendel and Wistar rats were used, and all the rats were maintained on a fox chow diet fed *ad libitum*.

In order to compare the enzyme systems in nuclei with those in the whole tissue, the enzyme systems were studied both in whole liver tissue and in isolated nuclei of the liver cells.

*Preparation of Suspensions of Whole Tissue*—The animals were killed by decapitation and the blood was drained from them as completely as possible. The blood remaining on the livers was washed off with saline and the livers were cut into small pieces. The small pieces were ground with 0.9 per cent saline in a glass homogenizer into a homogeneous, cell-free suspension. The suspension was passed through cheese-cloth to remove fiber. The time from killing the animals to starting the determination of oxygen consumption was limited to 30 minutes. For determinations of dry weight,

1 cc. of the suspension was dried in a weighed crucible to constant weight at 105° in an electric oven.

*Preparation of Isolated Cell Nuclei from Rat Livers*—The nuclei of rat liver cells were prepared by Dounce's method (5) at pH 6.0 to 6.2.

*Preparation of Coenzyme of d-Amino Acid Oxidase*—The coenzyme of d-amino acid oxidase was prepared from bakers' yeast according to the method of Warburg and Christian (6).

*Determination of d-Amino Acid Oxidase Apoenzyme*—The d-amino acid oxidase apoenzyme was determined by use of a Warburg apparatus. The procedure of Klein (7) was followed except that air was used instead of pure oxygen. The total volume of the solutions in the vessels was 2 cc. in all experiments. The oxygen uptake was recorded at 10 or 15 minute intervals during a period of 1 hour. dl-Alanine was used as substrate for all the determinations, since the oxidation of l-alanine under the conditions of experiment has been shown by Krebs (8) to be negligible. The controls without the amino acid were carried out under the same conditions at the same time. The substrate and coenzyme, if any was added, were both dissolved in pyrophosphate buffer at pH 8.3.

Addition of coenzyme did not increase the activity of preparations of whole liver, but preparations of isolated nuclei were found to be deficient in coenzyme. Therefore in all experiments with isolated nuclei, the above preparation of coenzyme from bakers' yeast was added in sufficient amount to assure maximum enzyme activity.

*Determination of Uricase*—This enzyme was determined by the method of Davidson (9) as modified by Elvehjem and his associates (10). The determinations were carried out at 37°, and the oxygen uptake was measured at 10 or 15 minute intervals for 1 hour. The results are expressed as the oxygen uptake per hour per mg. of dried tissue.

The inhibition of uricase by KCN has been used to confirm the presence of the enzyme.

Since the metal zinc, thought by some to be necessary for uricase action (10), might be removed during the process of preparing nuclei, zinc ions were added to the suspension of nuclei in concentrations employed by Wachtel, Hove, Elvehjem, and Hart (10), but no activating effect was found.

*Determination of Choline Oxidase*—The choline oxidase in whole liver and isolated liver nuclei was determined by use of a Warburg apparatus, according to the method of Mann and Quastel (11). The oxygen uptake caused by the oxidation of 1 mg. of choline hydrochloride by brei of normal rat liver or by isolated liver nuclei was measured at 10 or 15 minute intervals for a period of 1 hour.

*Number of Determinations Carried Out for Each Average Value of Enzyme Activity Reported in This Paper*—A total of five or six animals was used in

each experiment, which was performed in duplicate or triplicate. Any results which were not in agreement within 3 per cent were discarded. The nuclei of liver and tumor cells were prepared from 100 gm. of liver or tumor. Three to four experiments were done in each case.

### Results

*d-Amino Acid Oxidase in Whole Liver Tissue and in Isolated Nuclei of Liver Cells*—The average increase in oxygen uptake in livers of normal Osborne-Mendel rats, caused by the oxidation of *dl*-alanine, is 2.32 c.mm. per hour per mg. of dried tissue, and that in Wistar rats is 2.20 c.mm. The nuclei isolated from liver cells of normal Osborne-Mendel rats contained *d*-amino acid oxidase, as shown by the oxygen uptake of the nuclei in the presence of *dl*-alanine. The oxygen uptake caused by the oxidation of *dl*-alanine in liver nuclei of normal Osborne-Mendel rats is 1.67 c.mm. per hour per mg. of dried tissue, which is 78 per cent of the total oxidation of *dl*-alanine in whole liver tissue, and that of the nuclei isolated from Wistar rats is 1.10 c.mm., which is 50 per cent of the total oxidation of *dl*-alanine in the whole liver tissue.

The oxygen uptake caused by the oxidation of *dl*-alanine in isolated nuclei of liver cells of normal Osborne-Mendel rats was raised from 1.67 to 4.05 c.mm. per hour per mg. of dried tissue by the addition of sufficient coenzyme preparation to insure maximum enzyme activity, while that of the whole liver tissue was raised only to an insignificant degree (from 2.32 to 2.48 c.mm.). Similar results were also observed with nuclei of liver cells of normal Wistar rats.

The above results indicate that the apoenzyme of *d*-amino acid oxidase is slightly higher in activity in isolated nuclei than in whole liver suspensions, while the coenzyme is considerably deficient in the nuclei.

*Uricase in Whole Liver Tissue and in Isolated Nuclei of Liver Cells*—The oxygen uptake caused by the oxidation of uric acid in liver tissue in normal Osborne-Mendel rats is 2.85 c.mm. per hour per mg. of dried tissue, while that in Wistar rats is 3.03 c.mm.

The uricase activity in the liver nuclei of normal Osborne-Mendel rats is 4.16 c.mm. per hour per mg. of dried tissue, while that of the nuclei isolated from liver cells of normal Wistar rats is 3.94 c.mm. Thus the value for uricase activity per dry weight of isolated nuclei is higher than that of the whole liver tissue. It is about 46 per cent higher in liver nuclei of Osborne-Mendel rats and about 30 per cent higher in Wistar rats.

*Choline Oxidase in Whole Liver Tissue and in Isolated Nuclei of Liver Cells*—The oxygen uptake caused by the oxidation of choline hydrochloride in the liver tissue of normal Osborne-Mendel rats is 2.42 c.mm. per hour per mg. of dried tissue, and that of Wistar rats is 1.89 c.mm.

In the nuclei isolated from liver cells of rats of both Osborne-Mendel and

Wistar strains no significant activity of choline oxidase could be detected. This might be due to washing out of a coenzyme during the process of isolating the nuclei. However, the addition of the boiled aqueous extracts of whole liver did not promote the oxidation of choline in nuclei; so that it seems more probable that the enzyme itself is lacking in the nuclei.

#### DISCUSSION

From the results of recent work (1-5) it now seems clear that a number of enzymes are present in cell nuclei. In this paper we have shown that both *D*-amino acid oxidase and uricase are present in isolated nuclei from liver cells of normal rats. The concentration of the apoenzyme of *D*-amino acid oxidase in whole rat liver and in nuclei isolated from the liver cells is probably almost the same, although the nuclei appear to have a lower coenzyme concentration. This might be an indication that during the preparation of nuclei some of the coenzyme has been removed, but it is also possible that the cell nuclei normally are low in coenzyme, since this coenzyme is rather firmly bound to protein, at least in kidney preparations, and therefore should not be easily washed out at the pH range employed in preparing the nuclei.

We cannot explain, for the time being, why the isolated nuclei of liver cells of normal rats show a higher activity of uricase per dry weight than whole liver tissue.

Although choline oxidase was found in whole liver tissue, it was absent in samples of isolated nuclei. Since the addition of the boiled aqueous extracts of whole liver did not increase the activity of choline oxidase in isolated nuclei, it is not likely that a coenzyme or activator of this enzyme system is removed during the process of isolating the nuclei. Therefore it is probable that choline oxidase is lacking in nuclei of rat liver cells. The possibility that the enzyme is completely destroyed during preparation of the nuclei appears remote. •

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#### SUMMARY

1. An investigation has been made of the concentrations of *D*-amino acid oxidase, uricase, and choline oxidase in normal rat liver and in nuclei isolated from liver cells of normal rats.

2. It has been found that both *D*-amino acid oxidase and uricase are

present in nuclei of liver cells of normal rats. The concentration of uricase in nuclei is somewhat higher than that in whole tissue, while the concentration of *d*-amino acid oxidase apoenzyme is about the same in isolated nuclei and whole liver tissue.

3. The coenzyme of *d*-amino acid oxidase appears to be very deficient in isolated nuclei, whereas sufficient is present in whole tissue for optimal activity of the enzyme.

4. Choline oxidase was not found in isolated nuclei of liver cells. The addition of boiled aqueous extracts of whole liver did not promote the oxidation of choline in the isolated nuclei. This enzyme is present in whole liver tissue however.

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# THE DISTRIBUTION OF WATER, NITROGEN, AND ELECTROLYTES IN SKIN

BY LILLIAN EICHELBERGER, C. WESLEY EISELE, AND  
DOROTHY WERTZLER

(From the Department of Medicine of the University of Chicago, Chicago)

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A number of studies in this series have already been reported covering the distribution of water and electrolytes between blood and several of the body tissues; namely, muscle, kidney, and liver. The intention of this investigation is to define values for the distribution of water and electrolytes between the serum and skin of the dog under normal conditions, and also under several experimental conditions. This report, therefore, gives data on four groups of dogs: (1) a group of ten normal dogs, (2) a group of three pregnant dogs, (3) a group of six dogs with experimental renal hypertension, and (4) a group of eight hypertensive dogs having an increased total body water produced by the injection of isotonic salt solutions.

A number of investigators have reported the mineral content of mammalian skin. Since their data were calculated on the basis of either dry or wet skin as removed from the animal and not on the basis of fat-free skin, their results gave too large a deviation. Had the concentrations been expressed on a fat-free basis, the variation from animal to animal would have been much smaller. For that reason, the following references are only minutely serviceable. In 1912 Luithlen (1) reported the content of bases in rabbit skin; in 1920 Klose (2) published data on the water, sodium, potassium, and chloride content of the skin of normal infants; in 1924 McLaughlin and Theis (3) contrasted the mineral composition of the skin of the cow, calf, steer, bull, heifer, and goat; in 1926 Brown (4) prepared data on the calcium, magnesium, sodium, and potassium content in the skin of man, dog, and rabbit; in 1928 Nathan and Stern (5) recorded results on the mineral content of human skin; in 1942 Cornbleet, Ingraham, and Schorr (6) gave some data on potassium, calcium, and sodium in human skin.

Few workers have been interested in determining the electrolyte and water equilibria in the skin of animals. Recently, Manery and Hastings (7), working on soft tissues from the rat and the rabbit, concluded that skin belonged to the group of tissues in which Na:Cl ratios were the same as in an ultrafiltrate of plasma. A further characterization of mammalian skin was obtained by Manery *et al.* (8, 9) by using radioactive sodium and chloride. They found that the radioactive isotopes penetrated skin rapidly, becoming distributed between the plasma and the tissue to the same



magnitude as the normal isotope. Since these findings on skin were the same as for muscle, liver, and kidney, skin was incorporated in this group of tissues and, therefore, it was deduced that skin must contain three chemical phases: the extracellular phase which is in ionic equilibrium with plasma and consists essentially of plasma ultrafiltrate and connective tissue proteins, an intracellular phase which contains neither sodium nor chloride, and an intracellular phase which contains chloride but not its equivalent of sodium.

The many different functions of the skin are the product of a structure and chemical composition that are very complex, variable, and selective. This variation extends to the kind of animal, its age and habits, and even to the location of the skin on the animal. For this reason, it is unsound to attempt to draw any general conclusions about skin composition from the study of a single species

### *Procedure*

The normal dogs were kept for 3 weeks in metabolism cages on an alternate meat and Globe dog chow diet.<sup>1</sup> The pregnant animals were kept during the latter part of their pregnancy in the same way. The renal hypertension in the animals was produced by the constriction of renal arteries according to the Goldblatt technique (10). The dogs in this group were tractable animals weighing from 9 to 13 kilos and having a control mean blood pressure between 110 and 140 mm. of Hg. The mean blood pressure was recorded on a kymograph by means of a mercury manometer connected through a large glass cannula with an 18 gage needle which was introduced, by direct puncture, into a femoral artery. A 2.5 per cent sodium citrate solution was used as the anticoagulant in the system. After the constriction of the renal arteries, the blood pressure of the dogs was taken at weekly intervals. Dogs developing a mean blood pressure of 180 to 200 mm. of Hg were used for these experiments.

### *Methods*

For the removal of tissue, all animals were anesthetized with nembutal. The chest and abdomen were clipped with electric clippers and then shaved with a straight edge razor to remove as much hair as possible. After the shaving of the skin, it was washed with distilled water and dried with gauze. Blood was drawn under oil from the femoral artery and allowed to clot. The serum was used for the analyses of water, chloride, sodium, potassium, calcium, magnesium, and total nitrogen. Immediately after the withdrawal of blood the skin was dissected from the ventral wall of the abdomen and chest and placed in a glass-stoppered weighing bottle. An area of

<sup>1</sup> Globe Dog Chow Blox.

about  $10 \times 8$  cm. was removed. All incisions were closed with hemostats. The dogs in which an increased total body water was produced received an intravenous injection of 170 cc. per kilo of body weight of an isotonic salt solution containing 25 mm of  $\text{NaHCO}_3$  + 129 mm of  $\text{NaCl}$ . The salt solution was warmed to  $38^\circ$  and injected by gravity through the femoral vein at the rate of 45 cc. per minute. 30 minutes following the injection, a strip of skin approximately  $10 \times 8$  cm. on the opposite side of the mid-line was removed and a second sample of blood withdrawn from the femoral artery.

Each strip of the removed skin was quickly wiped with gauze, placed on a tile, and trimmed to remove any muscle and as much free fat as possible. It was then wiped again, cut into small strips approximately  $0.5 \times 2$  mm., and placed in a weighed glass-stoppered weighing bottle. To make sure that only the skin layers were being analyzed, one of the small strips was placed in formaldehyde for histological examination. The others, after being weighed, were placed in an oven at  $103^\circ$  and dried to constant weight. The dried skin was then extracted for neutral fat (11).

#### *Preparation of Skin Tissue for Electrolyte Determination*

Structures comprising the skin may be divided into two groups: (1) epidermis and corium with the subcutaneous connective tissue and blood vessels, lymphatics, and nerves and (2) the appendages of the skin embracing the hair follicles and special nerve endings. Owing to this complex structure, it was necessary to work out a method for the treatment of the tissue for chemical analyses. Some analyses were made on skin cut into small strips, but this procedure was not satisfactory because in cutting small strips of skin, more of one layer would be obtained in one strip than in another, thus giving widely deviating results. Another difficulty was the elasticity of the skin. To overcome this a large strip was quickly frozen with carbon dioxide snow and then cut with a razor blade into small strips. Again the results deviated disappointingly. The method finally developed was as follows: After determinations of water and neutral fat content had been made on the combined small strips of skin, they were transferred quantitatively to a special apparatus and pulverized (11-13). The pulverization of the skin tissue required a number of smashings. After all the smashed material was put through a No. 20 copper sieve, it was returned to a large agate mortar and ground to a homogeneous mixture. The mixture was then transferred to a weighing bottle and kept in a desiccator over sulfuric acid. Before each aliquot was removed for weighing the mixture was stirred thoroughly with a stainless steel spatula.

The following determinations were made on the skin: total fat, water, chloride, sodium, potassium, calcium, magnesium, total nitrogen, and collagen nitrogen. The chemical methods employed for these determinations

were the same as those previously reported (11), with the exception of the collagen method which will be discussed. The approximate amounts of the dry skin tissue used for the different determinations were, for chloride, 120 to 150 mg., for total nitrogen 50 mg., for collagen nitrogen 70 to 100 mg., for sodium and potassium 1 gm., and for calcium and magnesium 1.5 gm.

In a number of the experiments an attempt was made to determine the collagen of the skin by using a modification of the method of Lowry, Gilligan, and Katersky (14). When the dry, fat-free smashed skin was submitted to their method, the alkali-soluble fraction was so large that there was little collagen left to hydrolyze. This finding proved that the drying of the skin in an oven at 100° hydrolyzed most of the collagen to gelatin. Therefore, the method of Lowry *et al.* could not be used on dried skin. Since dehydrated tissue was used by Spencer, Morgulis, and Wilder (15) in their method for collagen determination in muscle, it was thought that their method would be applicable to skin dried at 100° for 48 hours. The limitations of this method are apparent. Too high results would indicate that some of the water-soluble proteins in skin (albumin) were being precipitated with the gelatin fraction. Too low results would indicate that some of the gelatin was further hydrolyzed as a result of the autoclaving. Since the centrifugate from the autoclaved skin gave no precipitate with 5 per cent trichloroacetic acid by the method of Janota (16), the albumin fraction of the skin must have been denatured by the previous drying of the skin, or precipitated, or broken down by the autoclaving at 20 pounds of pressure.

To check further the accuracy of the precipitation of gelatin by tannic acid, the centrifugates from a series of autoclaved skins were treated with 95 per cent alcohol by the method of Janota and the precipitated gelatin submitted to nitrogen determinations. Results obtained by both methods were alike.

The collagen determination was carried out as follows: 90 to 100 mg. of dried, fat-free, smashed skin were weighed into centrifuge tubes; 6 cc. of water were added to the powder, and the tubes covered with aluminum foil and allowed to stand overnight in the ice box. The tubes were autoclaved at 20 pounds of pressure for 4 hours, after which they were centrifuged and the centrifugate poured into 50 cc. centrifuge tubes containing 6 drops of concentrated hydrochloric acid. 5 cc. of 5 per cent tannic acid were then added. The skin residue was washed three times with hot water. The tubes were then placed in the ice box overnight, after which they were centrifuged. The precipitated gelatin was dissolved in 2 cc. of 2 *N* NaOH and transferred quantitatively to a Kjeldahl flask. The gelatin nitrogen was determined by the Campbell and Hanna method (17), the same as was used for the total nitrogen determinations. Collagen was estimated by multiplying the gelatin nitrogen value by 5.58. This factor of 5.58 was

obtained by numerous investigators (18-20) and is accepted by the packing industry.<sup>2</sup>

## DISCUSSION

*Values for Normal Skin*—The analytical data of the skins and serums from ten normal dogs are given in Tables I and II, together with the mean values with standard deviations.

TABLE I

*Analyses of Serum and Skin of Normal Dogs*

The values are given for fat-free skin.

Dog		pH	CO <sub>2</sub>	H <sub>2</sub> O	Cl	Na	K	Total N <sub>2</sub>	Colla- gen N <sub>2</sub>	Total fat
			<i>mm</i> <i>per l</i>	<i>gm.</i> <i>per kg</i>	<i>mm</i> <i>per kg</i>	<i>mm</i> <i>per kg</i>	<i>mm</i> <i>per kg</i>	<i>gm</i> <i>per kg</i>	<i>gm</i> <i>per kg</i>	<i>gm</i> <i>per kg</i>
O <sub>1</sub>	Serum	7 42	24 35	917 6	109 1	139 6	4 07	10 38		
	Skin			733 0	89 2	106 3	23 42	42 5	30 7	294
O <sub>2</sub>	Serum	7 42	25 40	916 2	106 7	140 2	3 87	10 45		
	Skin			717 6	87 0	98 4	18 27	43 0	29 5	284
O <sub>3</sub>	Serum	7 35	26 48	920 7	109.3	139.8	4 27	9 77		
	Skin			735 2	87 7	98 6	22 27	42 3	32 7	205
O <sub>4</sub>	Serum	7 33	25 64	920 1	107 9	139 3	3 66	9 74		
	Skin			725 8	87 3	93.6	26 07	44 2	33 1	160
O <sub>5</sub>	Serum	7 47	28 48	917 7	107 2	135 3	4 17	9 80		
	Skin			688 0	85 1	94 0	17 46	49 8	38.7	367
O <sub>6</sub>	Serum	7 42	28 21	922.6	102 0	139 0	3 97	9 56		
	Skin			705.4	81 9	91 9	26 31	46 4	30 7	350
O <sub>7</sub>	Serum	7 47	25 60	919 8	103 4	137 7	4 28	9 69		
	Skin			683.3	84 1	100 4	22 46	51 8	34 7	355
O <sub>8</sub>	Serum	7 47	25 51	920 9	111 0	139.0	4 99	9 26		
	Skin			703 0	86 9	93 5	22 98	48 1	32 4	223
O <sub>9</sub>	Serum	7 37	25 98	926 2	110 9	140 0	5 14	9 03		
	Skin			702 5	91 3	97 0	22 83	47 9	31 0	479
O <sub>10</sub>	Serum	7 50	21 90	921 6	108 3	138 5	3 40	8 91		
	Skin			689.4	86 1	90 9	22 35	51 7	37.2	320
Mean	Serum	7 42	25 75	920 3	107.6	138 8	4 19	9.66		
	$\sigma$	0.05	1 76	2 7	2 8	1 4	0 50	0.48		
	Skin			708 3	86 7	96 5	22 46	46 8	33.1	304
	$\sigma$			20 1	2 5	4 2	2 7	3 7	3.0	88

The water content and electrolyte concentrations of the skins from all the animals are consistently constant. This is attributable to the fact that all values are expressed in terms of fat-free skin (21). The large and variable percentage of fat in the skin is probably the result of the presence of some fat just beneath the corium layer, the large number of fat cells found in the reticular layer of dog skin, and the fat cells lining the hair follicles.

<sup>2</sup> Debeukelaer, F. L., Swift and Company, personal communication.

The greater portion of the solid matter of skin (292 gm. per kilo) is protein, mostly collagen. The total nitrogen value for skin was 46.8 gm. per kilo,  $\sigma$  3.7 gm., of which 33.1 gm. were collagen nitrogen. This amount of collagen nitrogen represents 185 gm.,  $\sigma$  17.4 gm., ( $5.58 \times 33.1$ ) of collagen per kilo of fat-free skin, leaving 107 gm. of solid matter to be allocated to the other proteins found in skin (mucins, albumin, globulin, melanin, keratins, elastins, etc.) Lowry *et al.* (14) studied the content of collagen in human skin and found an average value of 33.3 per cent of the wet weight of the tissue, which is nearly twice the value we found in dog skin. Our own preliminary studies on human skin give a collagen content more nearly that of dog skin.

The basic ion concentrations are very constant for the series. Potassium and magnesium concentrations are low in comparison with values found in other tissues, indicating indirectly a small intracellular phase; sodium and chloride values are high, indicating a large extracellular phase volume in skin.

TABLE II

*Calcium and Magnesium Content of Normal Skin*

The values are given in mm per kilo of serum and fat-free skin.

		Dog D <sub>6</sub>	Dog D <sub>7</sub>	Dog D <sub>8</sub>	Dog D <sub>9</sub>	Dog D <sub>10</sub>	Dog S <sub>1</sub>	Dog D <sub>16</sub>	Dog B <sub>9</sub>	Dog B <sub>10</sub>	Mean	$\sigma$
Ca	Serum	2 60	2 65	2 85	2 63	2 36	2 33	2 36	2 60	2 45	2 54	0 17
	Skin	2 81	3.25	2 71	2 87	2.59	2 70	3 00	4 40	2.74	3 01	0 52
Mg	Serum	1 02	1 05	1 08	1 12	1 03	1 02	0 76	1 14	0 82	1 01	0 12
	Skin	3.55	3 43	3 11	2 82	3 39	2.63	3 03	2 92	2 39	3.03	0.37

The conclusion of Manery, Danielson, and Hastings (22) that the extracellular phase of a tissue consists of extracellular fluid and connective tissue proteins is most applicable to the tissue skin. In the group of skin samples removed from normal dogs the connective tissue content of the skin was approximated from the collagen nitrogen by the method that Manery *et al.* (22) employed for muscle; that is, by assuming that the connective tissue of skin corresponds to tendon and that the ratio of collagen nitrogen to total nitrogen is the same in all connective tissue as it is in tendon. Since Manery *et al.* (22) assumed the ratio of collagen nitrogen to total nitrogen in the tendon to be the same for dog as for beef, the ratio was determined for dog tendon. Therefore, the Achilles tendons and tendons of the flexor and extensor muscles of the front leg were removed from dogs for analyses of total fat, water, total nitrogen, and collagen nitrogen. In these analyses there were wide variations in the content of the constituents in the different tendons, and also in different sections of each individual tendon. A summary of the data is given in Table III. The average total nitrogen content of 63.7 gm. per kilo of fat-free tendon, of which 95 per cent is

collagen nitrogen, differs from the values of the one beef tendon reported by Mitchell, Zimmerman, and Hamilton (23). The water content of these dog tendons is lower than those reported by Muntwyler *et al.* (24) but agrees both in average value and variation with the rabbit values reported by Manery *et al.* (22).

TABLE III  
*Water and Nitrogen Content of Dog Tendon*

This table represents the results obtained from twenty-one determinations. The values are expressed in gm. per kilo of fat-free tendon.

	Mean	$\sigma$	Maximum	Minimum
Fat	33	28	67	4.0
Water	613.1	25	656.5	563.0
Total nitrogen	63.7	6.3	74.7	51.9
Collagen nitrogen	60.6	5.4	68.4	50.4

TABLE IV  
*Estimated Weight of Connective Tissue in Skin with Calculated Concentrations of Sodium and Chloride in This Tissue*

The values are expressed in units per kilo of fat-free skin.

Dog No	Collagen N <sub>2</sub>	Connective tissue N <sub>2</sub>	Connective tissue weight	Connective tissue Cl	$\Delta$ Cl*	Connective tissue Na	$\Delta$ Na*
	gm	gm	gm	mm	mm	mm	mm
1	30.7	32.2	505	39.7	49.5	46.9	59.4
2	29.5	31.0	487	38.3	48.7	45.3	53.1
3	32.7	34.3	538	42.3	45.4	50.0	48.6
4	33.1	34.7	545	42.9	44.4	50.6	43.0
5	38.7	40.6	638	50.2	34.9	59.3	34.7
6	30.7	32.2	505	39.7	42.2	46.9	45.0
7	34.7	36.4	522	41.0	43.1	48.5	51.9
8	32.4	34.0	534	42.2	44.7	49.6	43.9
9	31.0	32.5	510	40.2	51.1	47.4	49.6
10	37.2	39.0	612	48.1	39.8	56.9	35.9
Mean	33.1	34.7	540	42.5	44.4	48.9	46.5
$\sigma$	3.0	3.1	46	3.6	4.5	4.5	7.4

\* Total skin concentration minus connective tissue concentration.

From the data in Table I, the weight of the connective tissue of skin was estimated, and then the amounts of chloride and sodium identified with this connective tissue were calculated from the analytical chloride and sodium data given by Muntwyler *et al.* (24). The chloride and sodium of the skin in excess of that accounted for by the connective tissue should represent the ultrafiltrate volume if there is no chloride or sodium in the intracellular phase. The results of these calculations are presented in Table IV. The

mean connective tissue phase consisting of the weight of the connective tissue plus the volume of ultrafiltrate as calculated from the chloride values ( $\Delta\text{Cl}$ ) amounted to 905 gm. per kilo of skin, whereas the mean connective tissue phase calculated from sodium values ( $\Delta\text{Na}$ ) amounted to 864 gm. The quantity of extracellular phase of skin thus calculated is so high that the intracellular phase is obviously too low when potassium and magnesium concentrations and total skin solids are considered. Assuming that the connective tissue of skin corresponds to tendon, the partition of collagen nitrogen to total nitrogen being 95 per cent, the connective tissue protein content of the skin from normal dogs averages 191 gm., leaving 101 gm. from the total solid content of skin (292 gm.) for the intracellular phase. Therefore, the intracellular phase must be much larger than the 101 gm. of its solid content. This indicates that some of the chloride and sodium of skin must be in the cells. This coincides with the reports of Amberson, Nash, Mulder, and Binns (25) and Manery and Hastings (7).

If all of the sodium and chloride of skin were extracellular and existed in the same proportion as in the ultrafiltrate of serum, the ratio of sodium to chloride in this phase of skin should approximate 1.26. Instead, in skin, a ratio of 1.11 was found, suggesting that the chloride and sodium of skin do not exist in the same proportion as was found in serum. This is shown by plotting the values for sodium and chloride of the skin from normal, pregnant, and hypertensive animals on a chart in which a solid line has been drawn representing an ultrafiltrate of serum (Fig. 1). It will be noted first that the skin values do not fall on the line but to the left of the line, indicating that skin must contain cells in which the intracellular chloride exceeds the intracellular sodium and, secondly, that a linear relationship does exist between the sodium and chloride concentrations in all of the skins.

*Values for Skin from Pregnant Dogs*—The results obtained from the analyses of skins and serums from three pregnant dogs are presented in Table V, along with the means and with standard deviations. A comparison of the mean values of skin from normal animals (Tables I and II) with those from pregnant animals shows an increased water content and a decreased total nitrogen content, the electrolyte concentrations being approximately the same. Regrettably, skin collagen was not determined on this group, and since the total water of the skin from pregnant dogs is higher than that of normal skin, we had no way to decide definitely in which phase this extra water had accumulated. If the potassium and magnesium of skin are attributed mostly to the intracellular fluids, the total content of potassium or magnesium should indicate the magnitude of the intracellular fluid. In analyses of the whole skin tissue, the potassium and magnesium contents per kilo of skin were found to be 21.97 mm,  $\sigma$  2.0, and 2.62 mm,  $\sigma$  0.25, respectively, which is the same concentration as that found in the skin

from normal dogs. Therefore, this indicates the extra water in the skin from pregnant dogs is probably in the extracellular phase. The interesting fact in this is that the pregnant animal has extra water stored in the skin.

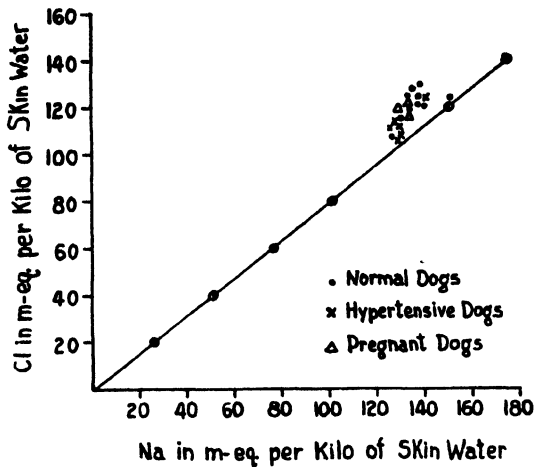


FIG. 1. The chloride of skin expressed in milliequivalents per kilo of skin water is plotted against the sodium of skin expressed in milliequivalents per kilo of skin water in all dogs. The values were calculated from Tables I, V, and VI.

TABLE V

*Analyses of Serum and Skin of Pregnant Dogs*

The values are given for fat-free skin.

Dog		pH	CO <sub>2</sub>	H <sub>2</sub> O	Cl	Na	K	Ca	Mg	Total N <sub>2</sub>	Fat
			<i>mM per l</i>	<i>gm per kg</i>	<i>mM per kg</i>	<i>mM per kg.</i>	<i>mM per kg.</i>	<i>mM per kg</i>	<i>mM per kg</i>	<i>gm per kg</i>	<i>gm per kg.</i>
D <sub>14</sub>	Serum			918.3	107.7	137.9	5.45	2.46	0.89	11.98	
	Skin			726.8	83.3	96.1	23.65	2.70	3.55	43.4	171
D <sub>16</sub>	Serum	7.31	23.05	930.3	110.8	138.6	4.14	2.43	1.17	8.14	
	Skin			770.5	91.3	100.5	23.15	2.49	1.96	36.4	120
D <sub>17</sub>	Serum	7.35	21.7	934.7	112.3	145.8	4.81	2.47	1.12	7.37	
	Skin			759.0	91.9	100.6	19.12	2.82	2.35	37.00	291
Mean	Serum			928.0	110.2	140.8	4.80	2.45	1.06	9.16	
	$\sigma$			8.0	1.8	3.5	0.51	0.02	0.12	2.00	
	Skin			752.1	88.8	99.1	21.97	2.67	2.62	38.9	
	$\sigma$			18.7	3.7	2.1	2.03	0.13	0.25	3.1	

*Values for Skin from Hypertensive Dogs*—In Table VI are presented detailed analytical data for six of the eight hypertensive animals. It will be seen that there is more variation in the concentration of constituents in



the serum of these animals than in normal animals. When the findings on skin from the hypertensive dogs are compared with those from normal dogs, the greatest and most consistent differences are an increased total water content with a decreased total nitrogen content. The increase in the total water content of 4 per cent is worth emphasis. Because the collagen method had not been worked out at the time that these experiments were carried out, the collagen nitrogen and therefore the connective tissue were

TABLE VI  
*Analyses of Serum and Skin of Hypertensive Dogs*

The values are given for fat-free skin.

Dog	Weight		Blood pressure	pH	CO <sub>2</sub>	H <sub>2</sub> O	Cl	Na	K	Ca	Mg	Total N <sub>2</sub>	Fat
	kg		mm Hg		mm per l	gm per kg	mm per kg	mm per kg	mm per kg	mm per kg	mm per kg	gm per kg	gm per kg
S <sub>3</sub>	12	Serum	180	7 33	24 45	926 0	112 2	145 1	3 76	2 46	1 08	9 79	
		Skin				722 0	86 9	99 8	21 0	2 22	3 49	45 3	7.4
S <sub>4</sub>	10 6	Serum	176	7 37	24 85	926 0	103 0	139 0	3 67	2 31	0 73	9 34	
		Skin				738 2	82 5	93 6	22.25	2.17	2 10	45 6	72 1
S <sub>7</sub>	11 2	Serum	180	7 33	29 20	920 6	106 6	133 5	3 60	2 45	1 05	9 80	
		Skin				757 0	81 3	98 6	23 85	2 62	2 33	38 25	72 6
S <sub>8</sub>	9 6	Serum	190	7 33	27.30	924 9	107 7	139 6	4.12	2.22	1 96	9 49	
		Skin				760 5	84.0	99 8	25 05	2 73	2 53	37 6	94 2
S <sub>2</sub>	11 4	Serum	175	7 33	27 25	918 2	115 2	142 9	3 95	2 53	0 85	10 66	
		Skin				750 2	81.5	98 6	27 30	2 72	2 47	39 5	210 6
S <sub>6</sub>	10 9	Serum	200			924.2	112 1	142.7	3 70	2 45	0 89	9 96	
		Skin				754 9	87 0	98 3	26.02	2 31	3 19	39 2	159 7
Mean		Serum				923.2	109.5	140.5	3.80	2.40	1.09	9.84	
		σ				2.9	4.1	3 7	0 18	0 10	0 40	0 42	
		Skin				747.1	83.9	98 1	24.58	2 46	2 69	40.9	
		σ				13.4	2 3	2.1	2.18	0.16	0.49	3 7	

not estimated in these skins. The outstanding finding in the skins of dogs with hypertension is the change in total water and nitrogen content, without changes in sodium and chloride concentrations. Since the potassium and magnesium concentrations did not change and since the osmotic pressure of intracellular fluids is largely determined by the concentration of these ions, it seems likely that the increase in the water did not come from the skin cells but from the vascular bed.

*Electrolyte and Water Content of Skin from Hypertensive Dogs before and after Injection of Isotonic Salt Solutions*—A study was made on the effect of an increase in total body water produced by the intravenous injection of 170 cc. per kilo of body weight of an isotonic salt solution containing 129 mm of NaCl + 25 mm of NaHCO<sub>3</sub>. Table VII gives one representative experiment from the group of eight dogs, together with the mean values with standard deviations from all of the eight dogs.

Since the skin from pregnant and hypertensive dogs contained larger amounts of water than that from normal dogs, we questioned whether it was possible for the skin to accumulate more water when the total body water was

TABLE VII

*Hypertensive Animals. Changes in Blood and Skin after Injection of Normal Isotonic Sodium Chloride Solution, 129 mm of NaCl + 25 mm of NaHCO<sub>3</sub>*

Dog S<sub>2</sub>; weight 11.4 kilos; 1938 cc. injected. Blood pressure, 180 mm.

		pH	CO <sub>2</sub>	H <sub>2</sub> O	Cl	Na	K	Ca	Mg	Total N <sub>2</sub>
			<i>mM</i> <i>per l</i>	<i>gm</i> <i>per kg</i>	<i>mM</i> <i>per kg</i>	<i>mM</i> <i>per kg</i>	<i>mM</i> <i>per kg</i>	<i>mM</i> <i>per kg</i>	<i>mM</i> <i>per kg</i>	<i>gm</i> <i>per kg</i>
Serum	Initial	7 33	27 25	918 2	115 2	142 9	3 95	2 53	0.85	10.45
	Final	7.31	26 85	945 4	116 8	146 5	2 98	1 63	0.64	6.43
Skin	Initial			750.2	81 2	98 6	27 3	2 72	2 47	39 7
	Final			824 0	92 2	112 0	20 4	1 91	1.75	27 0

Mean of 8 dogs

Serum	Initial	7 33	26 64	921 7	108 1	140 5	3 97	2 46	1 05	9.75
	$\sigma$	0 07	1 53	4 0	3 6	3 8	0 29	0 11	0 16	0.49
	Final	7 41	25 35	949 4	116 4	144 7	3 32	1 70	0 52	6 01
	$\sigma$	0 13	1 51	5 4	2 9	4 1	0 25	0 27	0 07	0 96
Skin	Initial			748 7	83 4	97 7	24 4	2 18	2 37	40 4
	$\sigma$			15.0	2 3	2 7	2 5	0 55	0.41	3.0
	Final			788 7	91 2	105 1	20 0	1 91	1 96	33 0
	$\sigma$			23	2 5	3.7	2.8	0 51	0 40	4 0

increased. In some of the experiments, even though the original amount of water in the skin was 75 per cent, there was a rise to 82 per cent following the increase in total body water. This percentage increase is very high compared with the increase found in any other tissues in the body under like experimental conditions (21, 12). The distribution of this water cannot be definitely described at this time, but slightly lowered potassium concentration indicates that there was little swelling of the tissue cells, whereas the large increases in sodium and chloride concentrations indicate a large increase in the extracellular phase.

Since these large volumes of water can be held by the skin and not by

other tissues of the body, there is in these experiments direct evidence that skin is a reservoir capable of taking care of a very considerable part of any excess fluid added to the body.

#### SUMMARY

Procedures are presented for water, electrolyte, total nitrogen, and collagen nitrogen analyses of whole skin.

Blood and skin taken simultaneously have been analyzed for total water and electrolyte concentrations from normal, pregnant, and hypertensive dogs. The results have been discussed.

For skin from normal dogs the means were as follows: total water,  $708.3 \pm 20.1$  gm.; chloride,  $86.7 \pm 2.5$  mm; sodium,  $96.5 \pm 4.2$  mm; potassium,  $22.4 \pm 2.7$  mm; calcium,  $2.54 \pm 0.17$  mm; magnesium,  $3.03 \pm 0.37$  mm; total nitrogen,  $46.8 \pm 3.7$  gm.; collagen nitrogen,  $33.1 \pm 3.0$  gm. per kilo of fat-free tissue.

By applying methods now accepted as valid for calculating the connective tissue proteins in tissue the weight of connective tissue in skin was estimated and found to be  $540$  gm.  $\pm$   $46$  gm. per kilo of fat-free skin. The possibility that the chloride in the skin tissue is not all extracellular has been considered and by inference under the conditions of these experiments there is indication that some of the chloride is within the cells. This conclusion was based on the following. (1) The Na:Cl ratio in the skin was found to be 1.11 instead of the ratio found in the plasma ultrafiltrate. (2) The intracellular phase calculated from the assumption that all of the chloride is extracellular either in the connective tissue or the ultrafiltrate was too low if the potassium and magnesium of skin are to be attributed to the intracellular space.

Experiments on hypertensive dogs are described in which the analyses of skin were carried out after an increase in the total body water had been produced by the injection of large volumes of isotonic salt solution. The increase in the relative percentage of total water of the skin amounted to a mean of 4 per cent which is actually 10 per cent higher than the normal skin value. It is apparent, therefore, that skin can be classed as a reservoir capable of taking care of a large part of any excess fluid added to the body.

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# THE EFFECT OF CYTOCHROME OXIDASE INHIBITORS ON THE FORMATION IN VITRO OF THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE WITH RADIOACTIVE IODINE AS INDICATOR\*

By H. SCHACHNER, A. L. FRANKLIN, AND I. L. CHAIKOFF

(From the Division of Physiology, University of California Medical School, Berkeley)

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The mechanism for the formation of thyroxine and diiodotyrosine in the thyroid gland is at present unknown. Some years ago Harington and Barger proposed that thyroxine is formed in the gland by the coupling of 2 molecules of diiodotyrosine (1). Since that time it has been demonstrated by several workers that such a reaction can occur. Von Mutzenbecher isolated thyroxine after incubating a basic solution of diiodotyrosine at 37° for long periods (2). This finding has been confirmed by Block (3) and also by Johnson and Tewkesbury (4). Von Mutzenbecher further showed that the synthesis of thyroxine under these conditions was inhibited by sodium sulfite and therefore he believes that oxidative steps are involved in this synthesis, the oxidizing agent being iodine or hypiodite split off from the diiodotyrosine. That hypiodite plays a rôle in the formation of thyroxine by the coupling of 2 diiodotyrosine molecules has been experimentally verified by Johnson and Tewkesbury (4); these workers found that hypiodite increased the amount of thyroxine formed. They also proposed a specific reaction mechanism involving oxidative steps to explain the formation of thyroxine from 3,5-diiodotyrosine.

It is shown here that oxidative steps participate in the formation of thyroxine and diiodotyrosine by thyroid tissue. This was demonstrated by a procedure in which the incorporation of labeled inorganic iodide into thyroxine and diiodotyrosine by surviving slices of sheep thyroid gland was measured (5).

## EXPERIMENTAL

*Preparation of Slices*—Thyroid glands from sheep weighing approximately 30 kilos were used in these experiments. The method of obtaining the thyroid glands and the preparation of slices of this tissue have been described (5). The preparation of the bicarbonate-Ringer's solution containing radioactive iodide and the method of incubation of thyroid slices

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at 38° were the same as those recorded elsewhere (5). 300 mg. of thyroid slices were transferred to each reaction flask, which contained 3.0 cc. of the bicarbonate-Ringer's solution. The vessels were gently agitated during the entire period of incubation.

*Addition of Inhibitors*—Azide, cyanide, and cysteine were added as solutions of  $\text{NaN}_3$ ,  $\text{NaCN}$ , and cysteine hydrochloride; 0.1 cc. of each was added to the flasks. These solutions were of sufficient strength to yield the concentrations recorded in Tables I to III when added to 3.0 cc. of the bicarbonate-Ringer's solution. The effect of the addition of the small volumes on the tonicity and volume of the medium was considered negligible. The reagents used were all of c.p. grade. Before their transfer to the flasks, the solutions of azide, cyanide, and cysteine hydrochloride were adjusted to pH 7.4.

A stock solution of  $\text{H}_2\text{S}$  was prepared by saturating distilled water with this gas at room temperature. Titration of an aliquot of this solution with base showed it to be about 0.1 M  $\text{H}_2\text{S}$ . The pH of the stock solution was adjusted to 7.4; 0.1 cc. of the solution was then transferred to the reaction flask containing 3.0 cc. of the bicarbonate-Ringer's solution.

*Anaerobic Conditions*—In one set of experiments anaerobic conditions were obtained by carrying out the experiments in the presence of a commercial gas mixture containing 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$  instead of the 95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$  mixture used in the control experiments. The bicarbonate-Ringer's solution was saturated with the former gas mixture, which contained a maximum of 0.6 per cent oxygen as an impurity. This same gas mixture was blown into the flask before and after the addition of the bicarbonate-Ringer's solution and again immediately after the slices were added.

In another set of experiments the small quantity of  $\text{O}_2$  present in the gas mixture was further reduced by suspending yellow phosphorus in a glass cup in the middle of the reaction flask (6). In this set of experiments, as well as in the previous one, the tightly stoppered reaction flasks were sealed with paraffin after the thyroid slices had been added and the atmosphere above the bicarbonate-Ringer's solution had been displaced with the  $\text{N}_2\text{-CO}_2$  mixture.

*CO Experiment*—Carbon monoxide was made by the action of hot concentrated sulfuric acid on formic acid. The method by which the gas mixture was prepared and introduced into the reaction flask has been described elsewhere (7). Dark conditions were obtained by completely covering the reaction flasks with black paint. In order to obtain light of sufficient intensity for the experiments described in Table V as "in light," the reaction flasks were exposed to the light from a 150 watt bulb.

*Separation of Iodine Compounds*—The fractionation procedure employed

for the separation of thyroxine, diiodotyrosine, and inorganic iodide was a modification of Blau's method, described elsewhere (5). Only in a few samples were radioactive thyroxine, diiodotyrosine, and inorganic  $I^{131}$  determined separately. In most experiments only thyroxine  $I^{131}$  and diiodotyrosine  $I^{131}$  were determined directly, and inorganic  $I^{131}$  was obtained by difference.

### Results

*Azide*—The effects of 0.005 M azide ( $NaN_3$ ) are shown in Table I. This substance reduced the percentage of the  $I^{131}$  recovered as thyroxine from

TABLE I

#### *Effect of Azide on Thyroxine and Diiodotyrosine Formation*

Each flask contained 300 mg. of slices of sheep thyroid in 3 cc. of a bicarbonate-Ringer's solution; period of incubation, 2 hours.

Experiment No		Per cent of Ringer's $I^{131}$ recovered as		
		Thyroxine	Diiodotyrosine	Inorganic iodine
1	Control	8.4	68.2	27.0*
		8.8	58.9	33.4*
		7.4	51.0	42.0*
	Azide, 0.005 M	0.4	10.3	89.3
		0.4	5.1	94.5
		0.7	10.9	88.4
2	Control	7.7	80.5	11.8
		6.0	83.4	10.6
		7.9	83.2	8.9
	Azide, 0.005 M	1.0	4.3	94.7
		0.9	6.6	92.5

\* In these experiments, each fraction was determined separately. The completeness of the recovery is shown by the sum of the values of the three fractions, namely 103.6, 101.1, and 100.4 per cent. Unless so designated, only thyroxine  $I^{131}$  and diiodotyrosine  $I^{131}$  were determined separately and inorganic  $I^{131}$  obtained by difference.

8 per cent to 0.4 to 1.0 per cent. The latter values show that the inhibition of thyroxine formation under the influence of azide is practically complete, since Morton *et al.* found amounts of similar magnitude in the thyroxine fraction when samples known to contain only inorganic  $I^{131}$  were analyzed by the procedure used here (8). In the same experiment it was shown that the amount of  $I^{131}$  recovered in the diiodotyrosine fraction did not exceed 1 per cent.

The degree of inhibition of diiodotyrosine formation produced by the azide was appreciable but in general was not as great as in the case of thyroxine formation.



*Sulfide*—The inhibition produced by sulfide was practically complete with respect to thyroxine formation (Table II). As in the case of azide, small amounts of diiodotyrosine were formed in the presence of 0.003 M  $H_2S$ . Since  $H_2S$  is a fairly strong reducing agent, it was desirable to show that its activity as an inhibitor was not due to its reducing power only. It can be shown that the reducing power of cysteine is comparable to that of  $H_2S$  (9, 10). For this reason the effect of cysteine upon the formation of thyroxine and diiodotyrosine by thyroid slices was tested.<sup>1</sup> Table II shows that the inhibition produced by cysteine is much smaller than that produced by  $H_2S$ . This result suggests that the inhibitory effect of  $H_2S$  is not due only to its reducing power.

TABLE II

*Effect of Sulfide and Cysteine on Thyroxine and Diiodotyrosine Formation*

Each flask contained 300 mg of slices of sheep thyroid in 3 cc. of a bicarbonate-Ringer's solution, period of incubation, 2 hours.

Experiment No		Per cent of Ringer's I <sup>131</sup> recovered as		
		Thyroxine	Diiodotyrosine	Inorganic iodine
1	Control	8.2	59.4	34.1*
	Sulfide, 0.003 M	0.4	3.4	96.2
		0.4	4.0	95.6
		0.4	4.6	95.0
		7.7	80.5	11.8
2	Control	6.0	83.4	10.6
		7.9	83.2	8.9
	Sulfide, 0.003 M	1.0	8.0	91.0
		1.4	4.6	94.0
	Cysteine, 0.02 M	3.0	71.4	25.6
		5.7	77.4	16.9

\* Control experiments are the same as those shown in Experiment 1 of Table I. Only average values are therefore given here.

*Cyanide*—The effects of cyanide on the thyroxine and diiodotyrosine formation were similar to those observed in the case of azide and sulfide (Table III).

*N<sub>2</sub> Anaerobiosis*—In Experiment 1 (Table IV) anaerobic conditions were produced by the use of a commercial gas mixture containing 95 per cent  $N_2$  and 5 per cent  $CO_2$ . A maximum of 0.6 per cent of oxygen was present in this gas mixture as an impurity. In the presence of so small an amount

<sup>1</sup> Unfortunately nothing is known about the comparative rates of the reductions effected by  $H_2S$  and cysteine in the thyroid gland. Hence the substitution of cysteine for  $H_2S$  as a reducing agent may not be completely valid. However, no other method of testing this point is available at present.

of oxygen the quantities of radioactive thyroxine and diiodotyrosine formed were not as great as in the controls, but the inhibitory effect of this degree of anaerobiosis was not as pronounced as the effects of cyanide, azide, and sulfide observed above.

In order to decide whether thyroxine and diiodotyrosine formation was completely independent of the presence of oxygen, the oxygen tension was reduced further by suspending yellow phosphorus in a glass cup in the middle of the reaction flask (6). This was done in Experiments 2 and 3. Under these conditions a further reduction in the amounts of radioactive thyroxine and diiodotyrosine formed was observed. But the amounts of thyroxine formed in these experiments were not negligible; this is par-

TABLE III

*Effect of Cyanide on Thyroxine and Diiodotyrosine Formation*

Each flask contained 300 mg of slices of sheep thyroid in 3 cc. of a bicarbonate-Ringer's solution, period of incubation, 2 hours

Experiment No		Per cent of Ringer's I <sup>131</sup> recovered as		
		Thyroxine	Diiodotyrosine	Inorganic iodine
1	Control	8.2	59.4	34.1*
	Cyanide, 0.01 M	0.2	9.9	89.9
		0.2	8.5	91.3
		0.2	7.3	92.5
2	Control	7.7	48.5	43.8
		7.2	53.6	39.2
		6.9	47.8	45.3
	Cyanide, 0.01 M	0.7	6.2	93.1
		0.7	16.1	83.2
		0.6	8.1	91.3

\* The control experiments are the same as those shown in Experiment 1 of Table I. Only average values are therefore given here.

ticularly well shown in the experiments of 3 and 4 hours duration, in which as much as 5 per cent of the I<sup>131</sup> was recovered as thyroxine.

*Carbon Monoxide*—Since the classical work of Warburg (11) CO has been extensively used in studies of biological oxidations (12, 13). The effects of CO upon thyroxine and diiodotyrosine formation in the dark as well as in the light are shown in Experiments 1 and 2 of Table V. In the former only traces of O<sub>2</sub> were contained in the flask; the gas mixture consisted of 95 per cent CO and 5 per cent CO<sub>2</sub>. In Experiment 2, 5 per cent O<sub>2</sub> was present; the gas mixture consisted of 90 per cent CO, 5 per cent CO<sub>2</sub>, and 5 per cent O<sub>2</sub>. Experiments in which the gas mixture in contact with the medium consisted of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub> were considered

adequate controls, since experiments recorded above on the effect of oxygen lack had shown that a great decrease in the  $O_2$  tension had only a small effect on the recovery of radiothyroxine and radiiodotyrosine.

TABLE IV

*Effect of Anaerobic Conditions on Thyroxine and Diiodotyrosine Formation*

Each flask contained 300 mg. of slices of sheep thyroid in 3 cc. of bicarbonate-Ringer's solution.

Experiment No.		Gas mixture above Ringer's solution			Period of incubation	Per cent of Ringer's $I^{131}$ recovered as		
		$O_2$	$CO_2$	$N_2$		Thyroxine	Diiodotyrosine	Inorganic iodine
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>hrs</i>			
1	Control	95	5	0	2	8.3	55.4	36.3
						7.6	45.6	46.8
						6.8	48.6	44.6
	$N_2$ , anaerobic		5	95*	2	5.6	53.0	41.4
						2.9	28.6	68.5
2	Control	95	5	0	2	4.6	37.9	57.5
						8.4	67.3	24.3
						8.6	60.2	31.2
	$N_2$ , anaerobic		5	95†	2	9.0	66.6	24.4
						2.2	21.9	75.9
						1.2	11.3	87.5
	Control	95	5	0	4	1.1	6.2	92.7
						9.5	67.9	22.6
						10.2	66.3	23.5
						5.1	47.6	47.3
3	Control	95	5	0	2	2.5	18.2	79.3
						9.7	74.7	15.6
						8.3	72.5	19.2
	$N_2$ , anaerobic		5	95†	2	8.9	74.3	16.8
						3.9	38.2	57.9
						3.6	35.6	60.8
	Control	95	5	0	3	2.8	24.7	72.5
						9.1	74.1	16.8
						9.3	67.9	22.8
	$N_2$ , anaerobic		5	95†	3	4.8	47.1	48.1
						2.6	66.6	30.8

\* A commercial preparation containing a maximum of 0.6 per cent  $O_2$ .

† Traces of  $O_2$  removed by suspending yellow phosphorus in the reaction flask.

A strong inhibition in the rates of thyroxine and diiodotyrosine formation is shown in Experiment 1. Under the conditions of this experiment, namely the almost complete absence of  $O_2$ , no pronounced difference was

observed in the recovery of radiothyroxine and radiodiiodotyrosine, whether the reaction was carried out in the light or in the dark.<sup>2</sup>

In Experiment 2, a distinct inhibition in the synthesis of radioactive thyroxine and diiodotyrosine was again observed, but in an atmosphere containing 5 per cent O<sub>2</sub>, the inhibitory effect of CO (particularly upon the diiodotyrosine formation) was less pronounced in the presence of light than in the dark. The difference observed in Experiments 1 and 2, namely

TABLE V

*Effect of Carbon Monoxide on Thyroxine and Diiodotyrosine Formation*

Each flask contained 300 mg. of slices of sheep thyroid in 3 cc. of bicarbonate-Ringer's solution; period of incubation, 2 hours.

Experiment No		Gas mixture above Ringer's solution				Per cent of Ringer's I <sup>131</sup> recovered as		
		O <sub>2</sub>	CO <sub>2</sub>	CO	Remarks	Thyroxine	Diiodotyrosine	Inorganic iodine
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	Control	95	5	0		12.1	51.9	36.0
						12.4	56.9	30.7
						11.5	47.0	41.5
	CO	Trace	5	95	In dark	1.4	6.0	92.6
						2.0	6.0	92.0
						3.9	11.5	84.6
	"	"	5	95	" light	3.4	6.8	89.8
						3.9	19.3	76.8
						2.6	7.8	89.6
2	Control	95	5	0		7.7	80.5	11.8
						6.0	83.4	10.6
						7.9	83.2	8.9
	CO	5	5	90	In dark	1.8	15.0	83.2
						2.6	21.0	76.4
						1.8	10.4	87.8
	"	5	5	90	" light	4.5	57.0	38.5
						4.8	54.5	40.7
						2.3	40.3	57.4

that light diminishes the CO effect when as little as 5 per cent O<sub>2</sub> is present but has no distinct effect in the absence of O<sub>2</sub>, is further evidence of the importance of oxygen in these reactions.

<sup>2</sup> In preliminary experiments it was also shown that the conversion of I<sup>131</sup> to thyroxine and diiodotyrosine in the controls (*i.e.* in the presence of a gas mixture consisting of 95 per cent O<sub>2</sub> and 5 per cent CO<sub>2</sub>) was the same irrespective of whether it took place in the light (150 watt bulb was used) or dark.

## DISCUSSION

At present only two enzymes, namely cytochrome oxidase and polyphenoloxidase, are known to be inhibited by *all four* of the following substances: KCN,  $\text{H}_2\text{S}$ , CO, and  $\text{NaN}_3$  (14). Polyphenoloxidase is found principally in plants and does not act on substrates usually present in animals (15). The inhibition of the incorporation of  $\text{I}^{131}$  into thyroxine and diiodotyrosine by each one of these poisons together with the marked effect of light on the inhibition by CO (this is perhaps the best single piece of evidence for this point (13)) shows clearly that the cytochrome-cytochrome oxidase system is involved in the formation of these iodine compounds by the thyroid gland.<sup>3</sup>

In view of this conclusion, it is somewhat surprising that a great reduction in the oxygen tension in the atmosphere in contact with the thyroid slices (from 95 to less than 1 per cent) had little effect on the conversion of inorganic  $\text{I}^{131}$  to thyroxine and diiodotyrosine when compared with those of azide, sulfide, and cyanide. That oxygen plays a rôle in the incorporation, however, was clearly shown in experiments in which the percentage of oxygen in the atmosphere was reduced below 0.6. Since it is difficult to remove the last traces of oxygen from the surviving tissue slices, it cannot be decided whether the formation of radiothyroxine and radio-diiodotyrosine observed under the conditions described in Experiments 2 and 3 of Table IV is due to reactions in which traces of oxygen participate or to reactions which do not involve oxygen. The latter assumption, however, does not invalidate the main thesis of this paper, namely that the cytochrome-cytochrome oxidase system is directly involved in the formation of thyroxine and diiodotyrosine by surviving thyroid tissue, since reduction of the oxygen content of the reaction flasks below 0.6 per cent did produce marked inhibition in the formation of thyroxine and diiodotyrosine.

Li (16) found that the rate of iodination of tyrosine to 3,5-diiodotyrosine is a function of the concentrations of hypiodous acid (HIO) and of iodine ( $\text{I}_2$ ). If a similar mechanism is involved in the formation of diiodotyrosine by animal tissues, then it is conceivable that the oxidation of iodide either directly or indirectly through the cytochrome-cytochrome oxidase system provides the HIO and  $\text{I}_2$  in the tissue. To this supposition the objection might be raised that  $\text{I}_2$  and HIO are not present in tissues in measurable amounts. But in a heterogeneous system such as that found in a cell

<sup>3</sup> Since cyanide, azide, and sulfide inhibit oxidative enzymes other than cytochrome oxidase (*e.g.* peroxidase), the possibility of the participation of oxidative enzymes other than cytochrome oxidase in the formation of thyroxine and diiodotyrosine is not ruled out here.

it is conceivable that, even though infinitesimal amounts of the substances are present, they are sufficiently concentrated at the site of reaction to iodinate tyrosine.

#### SUMMARY

The influence of anaerobiosis and of substances that inhibit cytochrome oxidase upon the incorporation of radioactive inorganic iodide into diiodotyrosine and thyroxine by surviving thyroid slices was investigated.

1. Cyanide, azide, sulfide, and carbon monoxide inhibited the formation of diiodotyrosine and thyroxine from inorganic iodide by thyroid tissue. The inhibition produced by carbon monoxide was more pronounced in the dark than in the light.

2. Anaerobiosis caused a strong inhibition when the amount of  $O_2$  was reduced below 0.6 per cent.

3. It is concluded that the formation of both diiodotyrosine and thyroxine by the thyroid gland is linked with aerobic oxidations in which the cytochrome-cytochrome oxidase system is involved.

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# IMPROVEMENTS IN THE LACTOBACILLUS CASEI ASSAY FOR BIOTIN\*

BY G. M. SHULL AND W. H. PETERSON

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

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Some investigators who have used our method for the determination of biotin (1) have informed us that they did not obtain satisfactory growth and acidity with even an excess of biotin. A few of the men in our own laboratory who have prepared the medium for the first time have also encountered the same difficulties. Whenever possible, we have obtained samples of these media, and have invariably found the trouble to be due to a low level of eluate factor (2, 3) in the medium. The main source of this factor is yeast extract, but as this also contains much biotin the problem encountered is to remove the biotin and still leave an adequate supply of eluate factor. It is necessary to remove most of the biotin rather than merely to inactivate it with peroxide because high concentrations of peroxide-treated biotin in the basal medium lead to unreproducible results. The difficulty experienced by some workers in this and other laboratories was caused by removal of too much eluate factor as well as biotin by the norit treatment given in the original method. The yeast supplement described below provides an optimal amount of eluate factor and only a small amount of peroxide-treated biotin.

*Preparation of Yeast Supplement*—20 gm. of yeast extract (Difco) are dissolved in a liter of water and the pH is adjusted to 2.0 with  $\text{H}_2\text{SO}_4$ . 4 gm. of norit A are added and the adsorption is carried out at 55–60° with mechanical stirring for 30 minutes. The norit is filtered off and the filtrate saved for later use. The norit is eluted with 200 ml. of 50 per cent ethanol at room temperature for 15 minutes. This eluate, which contains a part of the biotin of the original material, is discarded and the norit is eluted a second time with 200 ml. of a solution containing 10 parts of  $\text{NH}_4\text{OH}$  (28 per cent), 50 parts of 95 per cent ethanol, and 40 parts of water, at 55–60° for 15 minutes. The extracted norit is discarded and the eluate is distilled *in vacuo* to remove the  $\text{NH}_3$  and ethanol. The volume of the eluate is then made up to 200 ml. and the pH is adjusted to 3.0. 10 gm. of Supër Filtrol (Filtrol Corporation, Los Angeles) are added and the mixture is stirred for 15 minutes at room temperature. The Super Filtrol adsorbs the eluate factor readily but leaves most of the biotin in the filtrate. After filtration

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the Super Filtrol is eluted with 200 ml. of the ammoniacal ethanol solution described above for 15 minutes at room temperature. The eluate is distilled *in vacuo* until the  $\text{NH}_3$  and ethanol are removed. It is then added to the norit-yeast filtrate from the first operation and the volume of the combined solution is made to 1300 ml. The pH is adjusted to 3.0 and 13 ml. of superoxol (Merck, 30 per cent  $\text{H}_2\text{O}_2$ ) are added to oxidize the small amount of biotin contained in it. The solution is allowed to stand for 24 hours at room temperature. At the end of this period the pH is adjusted to 7.0 and 15 gm. of  $\text{MnO}_2$  are added with stirring. When the evolution of oxygen has ceased, the solution is filtered and is now ready for use in the assay.

Since different batches of yeast extract contain varying amounts of eluate factor, it is well to test each preparation to ascertain whether it contains an adequate amount of the factor. An assay should be set up in which all tubes contain 1 millimicrogram of biotin and varying amounts of the yeast supplement (from 0.5 to 3.0 ml.) are added per tube. A titration of about 11.0 ml. of 0.1 N alkali indicates that there is sufficient eluate factor present and somewhat more than the required volume of the supplement should be used subsequently in all biotin assays.

*Inoculum*—The inoculum previously described has proved satisfactory when uniform drops of it were used in the assay tubes. However, unless great care is taken, the size of the drops will vary and the possibility of getting more inoculum in one tube than in another becomes greater. An increased titer always resulted when more than 1 drop of this heavy inoculum was added per tube. By removing the suspending medium and diluting the resuspended cells, an inoculum which was independent of drop size or number of drops (1 to 3) was obtained. It is prepared in the following manner.

A transfer from the stock culture into 10 ml. of the yeast extract medium described in the procedure is made 1 full day before the assay is to be set up. After 20 to 30 hours incubation at  $37^\circ$ , the cells are centrifuged and resuspended in 10 ml. of sterile water. 1 drop (0.05 ml.) of the resuspended cells is transferred to 10 ml. of sterile water, and 1 drop of this dilute cell suspension is used in each assay tube for inoculum.

#### SUMMARY

Two modifications of the *Lactobacillus casei* assay for biotin are presented. The eluate factor level in the yeast supplement of the basal medium is increased by an elution procedure so that optimal growth of the organism is insured. The preparation of an inoculum which is independent of drop size is described.

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# STUDIES ON THE METABOLISM OF CLOSTRIDIUM TETANI

By M. J. PICKETT

(From the Department of Bacteriology and Immunology, Harvard Medical School and School of Public Health, Boston)

(Received for publication, September 9, 1943)

For the development of a synthetic medium which will support good growth of *Clostridium tetani*, and at the same time give good yields of toxin, it would appear desirable to have available further data on the metabolism of this organism. The amino acid requirements have recently been investigated by Mueller and Miller (1) and Feeney, Mueller, and Miller (2). They found that tryptophane, tyrosine, leucine, isoleucine, histidine, valine, and arginine were essential for growth in a synthetic medium, while threonine, serine, and phenylalanine were strongly stimulatory. Glutamic acid, although not stimulatory, permitted much heavier growth. Clifton (3) has studied the utilization of several of these amino acids, as well as possible intermediate products, by washed suspensions of *Clostridium tetani*. He found no CO<sub>2</sub> or NH<sub>3</sub> production from glycine, alanine, glycine plus alanine, and proline, whereas these two products were formed from glutamic and aspartic acids and from serine. Pyruvic and fumaric acids were also attacked, but not glycerol, glucose, and formic, acetic, butyric, lactic, maleic, malic, glycolic, and succinic acids.

This paper is concerned with further studies on the metabolism of amino acids and possible intermediate compounds by washed suspensions of *Clostridium tetani*. Four strains were employed in preliminary experiments by the Thunberg procedure and one of these, the Tulloch III strain, was used for more detailed studies on the metabolism of histidine and malic acid.

## EXPERIMENTAL

### *Methods*

1 liter of infusion broth containing 0.5 per cent glucose was inoculated with 1.0 ml. of a 24 to 48 hour thioglycolate culture of the organism and incubated anaerobically at 37°. The growth was harvested at 18 to 20 hours, washed once with physiological saline, and finally resuspended in saline.

The usual Thunberg technique was employed to detect hydrogen donors, and the Warburg technique to determine CO<sub>2</sub>, H<sub>2</sub>, and acid production. O<sub>2</sub>-free N<sub>2</sub> or H<sub>2</sub> served as the gas phase when phosphate buffer was used in the experiments by the Warburg method, and N<sub>2</sub> containing 5 per cent CO<sub>2</sub> when bicarbonate buffer was used. When chemical analyses were to be

made for the end-products of metabolism, 50 ml. of washed, buffered suspension containing the substrate under study were placed in a 125 ml. Erlenmeyer flask which was equipped for deaeration with  $O_2$ -free  $N_2$ . After incubation at  $37^\circ$  (several hours to several days), the suspension was acidified with 0.2 volume of 5 per cent metaphosphoric acid and the  $CO_2$  was collected by aeration in standard  $Ba(OH)_2$  solution, the amount of  $CO_2$  formed then being determined by back titration of the excess baryta with  $0.04\ N\ H_2SO_4$ . The suspension was then centrifuged and subsequent analyses were made on the protein-free supernatant fluid. Ammonia was determined according to Woods (4), fumaric and succinic acids according to Krebs, Smyth, and Evans (5), and lactic acid according to Friedemann and Graesser (6) save that the lactate was oxidized at  $60^\circ$  with 10 per cent  $Ce_2(SO_4)_3$  in normal  $H_2SO_4$  rather than at  $100^\circ$  with permanganate. Volatile acids in steam distillates were tentatively identified by Duclaux distillation, ether partition according to Osburn and Werkman (7), and ether partition of the first 60 ml. collected from Duclaux distillation. Alcohol was determined by oxidation with standard dichromate and titration of the excess dichromate with thiosulfate. Histidine and its derivatives were separated and determined according to Koessler and Hanke (8).

### Results

Since Clifton's (3) studies did not include experiments by the Thunberg procedure on individual amino acids, eighteen of these were tested, methylene blue being used as the hydrogen acceptor (Table I). No significant qualitative differences, in respect to dehydrogenases for the substrates tested, were observed among the four strains. Glutamic acid and threonine appeared to serve most readily as H donors, while serine and histidine reduced methylene blue somewhat more slowly, and methionine was the weakest H donor of those amino acids which were attacked. Of the non-amino compounds tested, only succinic acid failed to reduce methylene blue. Malic and lactic acids were rapidly oxidized by methylene blue in the presence of *Clostridium tetani*, although Clifton (3) failed to obtain evidence that these compounds are decarboxylated in the absence of an H acceptor. However, the possibility that malic acid might be decarboxylated slowly was further investigated.

Experiments carried out according to the Warburg procedure, with washed suspensions in phosphate buffer, of relatively long duration (4 hours) indicated that malic acid was slowly decarboxylated, the  $Q_o$ , being about 2. Similar experiments with bicarbonate buffer showed a decrease of 33 c.mm. of bound  $CO_2$ , thus indicating a slow production of acid. Addition of 0.02 per cent methylene blue to the vessels did not increase the rate of decarboxylation of the malic acid.

The fermentation of malic acid was studied in more detail in semimacro

experiments, 50 ml. of washed, buffered suspension being incubated for 6 to 8 days at 37° in the presence of 1 mM of sodium malate. The results obtained from a typical experiment are summarized in Table II. No corrections were made for the traces of compounds found in the control suspensions.

TABLE I

*Dehydrogenation of Various Substrates by Clostridium tetani*

The relative rates are given by (+++) = rapid reduction to (-) = no reduction of the methylene blue.

Substrate	Strain			
	Albany	Tulloch II	Tulloch III	Toronto
<i>dl</i> -Alanine	-	-	-	-
<i>d</i> -Arginine	-	-	-	-
<i>dl</i> -Aspartic acid	++	++	++	++
<i>d</i> -Glutamic "	+++	+++	+++	+++
Glycine	-	-	-	-
<i>l</i> -Histidine ..	++	+	++	++
<i>dl</i> -Leucine	-	-	-	-
<i>dl</i> -Isoleucine	-	-	-	-
<i>dl</i> -Lysine	-	-	-	-
<i>dl</i> -Methionine	+	+	++	+
<i>dl</i> -Phenylalanine	-	-	-	-
<i>l</i> -Proline	-	-	-	-
<i>l</i> -Hydroxyproline	-	-	-	-
<i>dl</i> -Serine	++	++	++	+++
<i>dl</i> -Threonine	+++	+++	+++	+++
<i>l</i> -Tryptophane	-	-	-	-
<i>l</i> -Tyrosine	-	-	-	-
<i>dl</i> -Valine	-	-	-	-
Glucose			++	
Glycerol			+++	
Lactic acid	+++	+++	+++	+++
Malic "	+++	+++	+++	+++
Succinic acid	-	-	-	-

Determinations of lactic, formic, fumaric, and malic acids made on ether extracts of the fermentation mixtures indicated that only the latter acid was present. Malic acid was determined manometrically by oxidation with 5 per cent ceric sulfate in 0.5 N H<sub>2</sub>SO<sub>4</sub>, 2 moles of CO<sub>2</sub> being formed per mole of malate. Lactate and oxalacetate are also oxidized by ceric sulfate; hence these compounds, if present, must be determined separately by more specific methods and the manometric results corrected accordingly. Formic acid was also determined manometrically by oxidation with 0.5 M

mercuric acetate in 0.1 N HCl. Acetone (10) and oxalacetate (11) were also sought but not found.

The ratio, succinic acid formed to malic acid fermented, was near 0.5 in all experiments, while the ratios for CO<sub>2</sub> and volatile acids increased somewhat with prolonged incubation of the suspensions. Variable, although always small, amounts of alcohols were found, and no attempt was made at their identification.

Attention was next directed toward further studies on the utilization of histidine by washed suspensions of *Clostridium tetani*. This amino acid, it will be recalled, was found by Feeney *et al.* (2) to be among those required for growth of this organism in a synthetic medium.

Histidine was fermented with rupture of the imidazole nucleus, the end-products being NH<sub>3</sub>, CO<sub>2</sub>, and volatile acids (87 per cent acetic, 13 per cent

TABLE II  
*Fermentation of Sodium Malate by Washed Suspensions of Clostridium tetani*  
(Tulloch III Strain)

	Product							
	Malic acid	CO <sub>2</sub>	Acetic acid	Butyric acid	Succinic acid	Alcohol*	Total	Theory
Found, mg	40.1	25.5	17.7	4.3	39.2	3.4	130.2	134.1
Yield, moles per 100 moles fermented		82.8	42.0	7.0	47.4	10.4		
Yield, mg. per mm fermented		36.4	25.2	6.2	56.0	4.8	128.6	134.1
C, mg. per mm fermented		9.9	10.1	3.4	22.8	2.5	48.7	48.0
H, " " " "			1.7	0.6	2.8	0.6	5.7	6.0
O, " " " "		26.5	13.4	2.2	30.4	1.7	74.2	80.0
Available H (Barker (9))							12.7	12.0

\* Reported as ethyl alcohol.

butyric). A trace of alcohol was also found but was not identified. Neither production nor consumption of hydrogen was observed in experiments done by the Warburg method with phosphate buffer and H<sub>2</sub> or N<sub>2</sub> as the gas phase. The optimal pH for both deamination and decarboxylation was studied over the range pH 5.8 to 8.1. The results (Fig. 1) were similar to those reported by Gale (12) for *Pseudomonas aeruginosa* and a paracolon bacillus in that decarboxylation proceeded most rapidly in a slightly acid medium, whereas deamination proceeded slowly below pH 6 but increased rapidly in more alkaline media to reach a maximum at about pH 7.5.

Other end-products of fermentation than those reported above were also sought. 1 mm of histidine was added to duplicate suspensions and the latter incubated for 24 hours at 37°. An aliquot of each suspension was then analyzed for imidazole derivatives according to Koessler and Hanke (8). Typical results are presented in Table III.

Neither methylimidazole nor histamine was found, whereas the ammonia plus the histidine fraction (calculated as histidine) accounted for all the

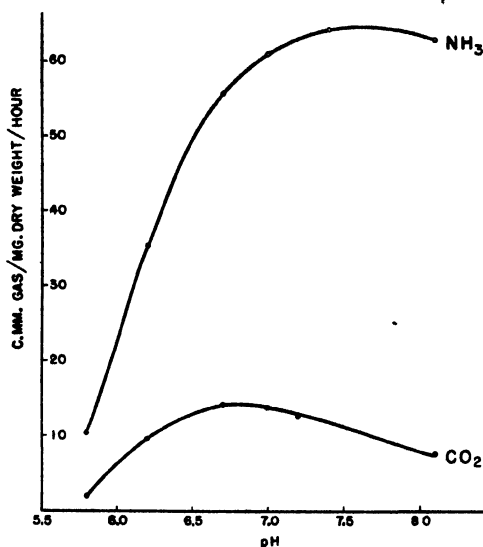


FIG. 1.  $Q_{NH_3}$  and  $Q_{CO_2}$  curves obtained from the fermentation of 2 ml of 0.01 M histidine by washed suspensions of *Clostridium tetani*.

TABLE III  
Fermentation of Histidine by Washed Suspensions of *Clostridium tetani*

	Product							
	Histi- dine	NH <sub>3</sub>	CO <sub>2</sub>	Acetic acid	Butyric acid	Alcohol	Total	Theory*
Found, mg	40.3	36.9	22.1	93.8	20.2	Trace		
Yield, moles per mole fermented		2.93	0.68	2.11	0.31			
Yield, mg. per mm fer- mented		49.8	29.9	126.7	27.3		233.7	231
C, mg. per mm fermented			8.2	50.7	14.9		73.8	72
H, " " " "		8.8		8.4	2.5		19.7	18
O, " " " "			21.7	67.6	9.9		99.2	99
N, " " " "		41.0					41.0	42
Available H							31.9	29

\* Corrections for additional water in the end-products were calculated from the oxygen recovered, and corresponded to histidine + 4.2H<sub>2</sub>O.

added nitrogen. That the histidine fraction contained only this amino acid, and no imidazolepropionic, imidazoleacetic, or imidazolelactic acid, was confirmed by determination of amino nitrogen by the Van Slyke

method. Incubation of the suspensions for 48 rather than 24 hours gave identical yields of ammonia, namely 2.2 rather than 3.0 mm, the theoretical yield for complete degradation of the histidine added, while slightly more CO<sub>2</sub> and less volatile acid were obtained from the 48 hour suspensions. The reason for this abrupt cessation in fermentation when only 73 to 75 per cent of the histidine had been decomposed was not investigated.

#### DISCUSSION

It is apparent that the growth requirements of *Clostridium tetani* include amino acids which, individually, are not readily attacked. Of the seven amino acids which are required for growth in a synthetic medium (1, 2), only histidine is dehydrogenated by washed suspensions of the organism. On the other hand those amino acids which stimulate or increase the total growth are, with the exception of phenylalanine, readily dehydrogenated. Further study may disclose that phenylalanine also belongs in one of these two groups: It may (1) partially replace or supplement tyrosine as an essential amino acid, or (2) be attacked slowly, even though experiments by the Thunberg procedure indicated that it is not readily dehydrogenated. From the evidence now available one is led to conclude not only that *Clostridium tetani* requires a number of preformed amino acids for growth, but also that the majority of these amino acids may be converted with little structural change into cell substance. The energy requirements, on the other hand, are not specific. Glutamic acid in a casein hydrolysate medium, for example, is undoubtedly the chief source of energy but other compounds such as serine or pyruvic acid should serve equally well.

While the essential amino acid, histidine, is fermented with rupture of the imidazole nucleus by washed suspensions of *Clostridium tetani*, it is unlikely that this occurs at a significant rate when other readily available energy-yielding compounds are present, since only a few micrograms per ml. are required for growth in a completely synthetic medium. It should be noted here, however, that histidine appears to be a purine precursor in mammalian metabolism (13). The presence of an enzyme system for histidine in the tetanus bacillus may, therefore, be more than fortuitous, since this amino acid may be a precursor in the synthesis of nucleoprotein.

Malic and succinic acids were included in this study because of their possible rôle as hydrogen carriers. Neither the presence of succinic dehydrogenase nor an accumulation of oxalacetate could be demonstrated, however; hence it is unlikely that a C<sub>4</sub>-dicarboxylic acid catalysis plays an important part in the metabolism of this organism. Furthermore the end-products obtained from the slow fermentation of malic acid do not support the assumption that malate is dismuted to succinic and oxalacetic acids, with subsequent degradation of the oxalacetate via pyruvic acid.

## SUMMARY

1. Histidine, methionine, serine, threonine, and aspartic and glutamic acids were shown to be dehydrogenated by washed cells of *Clostridium tetani*. Alanine, arginine, glycine, leucine, isoleucine, lysine, phenylalanine, proline, hydroxyproline, tryptophane, tyrosine, and valine were not dehydrogenated under comparable experimental conditions.

2. Histidine is fermented by washed suspensions of this organism with rupture of the imidazole nucleus, the chief end-products being carbon dioxide, ammonia, and acetic and butyric acids.

3. Malic acid is slowly fermented to carbon dioxide, alcohol, and acetic, butyric, and succinic acids.

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# MERCAPTURIC ACIDS

## I. THE SYNTHESIS OF PHENYL-*l*-CYSTEINE AND *l*-PHENYLMERCAPTURIC ACID

BY S. H. ZBARSKY AND LESLIE YOUNG

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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The fact that naphthalene (1, 2) and anthracene (3) are converted to S-substituted N-acetylcysteine derivatives, *i.e.* mercapturic acids, in the animal body raises the possibility that formation of phenylmercapturic acid takes place following the administration of benzene to animals. Various workers (4-6) have obtained evidence of mercapturic acid excretion by animals dosed with benzene, but none has described the isolation of phenylmercapturic acid from the urine of such animals. The present investigation of the synthesis of phenyl-*l*-cysteine and *l*-phenylmercapturic acid was undertaken as a preliminary step to a study of the metabolic formation of phenylmercapturic acid.

The only previously described preparation of phenylmercapturic acid was that of Baumann and Preusse (7). These workers obtained the compound by the debromination of *p*-bromophenylmercapturic acid isolated from the urine of dogs to which bromobenzene had been administered. The preparation of phenylmercapturic acid by this method was repeated in the present work and in addition the compound was synthesized by two methods which are more direct than that described by Baumann and Preusse. The first method was based on that described by Ing, Bourne, and Young (8) for the synthesis of *l*- $\alpha$ -naphthylmercapturic acid. Diazotized aniline was coupled with acetylcysteine and the product of this reaction was decomposed with copper powder to yield phenylmercapturic acid. In the second method phenylmercapturic acid was obtained by treating phenyl-*l*-cysteine with acetic anhydride. Phenyl-*l*-cysteine was prepared by two methods; the first was a modification of that used by du Vigneaud, Wood, and Binkley (9) to obtain *p*-bromophenyl-*l*-cysteine, and the second involved the debromination of *p*-bromophenyl-*l*-cysteine.

### EXPERIMENTAL

#### *Phenyl-l-cysteine*

*Method I*—The first method used for the preparation of phenyl-*l*-cysteine was based on that described by du Vigneaud, Wood, and Binkley (9) for the preparation of *p*-bromophenyl-*l*-cysteine. 3 gm. of zinc dust were added

to a solution of 5 gm. of *l*-cystine in 100 ml. of 1.5 *N* sulfuric acid. The mixture was heated on a water bath for 4 hours and small pieces of mossy zinc were added from time to time. At the end of this period the mixture was filtered while hot and an aqueous suspension of cuprous oxide was added with stirring to the filtrate until no more went into solution and the supernatant liquid became blue. The solution of cysteine cuprous mercaptide thus obtained was cooled in an ice bath. 4 gm. of freshly distilled aniline were diazotized in a sulfuric acid medium and the solution was added slowly with stirring to the ice-cold mercaptide solution. Stirring was continued for 45 minutes. The mixture was then heated for 20 minutes at 60–70° and filtered. The filtrate was extracted with two 50 ml. portions of ether. The aqueous solution was saturated with hydrogen sulfide and the precipitate of copper sulfide which formed was separated by filtration and washed twice with small portions of 1.5 *N* sulfuric acid. The washings were combined with the filtrate and excess hydrogen sulfide was removed from the solution by heating it to 50° under reduced pressure for 20 minutes. The solution was cooled and phenyl-*l*-cysteine was precipitated by making the solution neutral to Congo red by the careful addition of ammonium hydroxide. An examination of the precipitate suggested that it was contaminated with cystine. The precipitate was dissolved in 50 ml. of 2 *N* hydrochloric acid, granular tin was added, and the mixture was heated on the water bath for 3 hours and then filtered. The solution was saturated with hydrogen sulfide, filtered, and excess hydrogen sulfide was removed from the filtrate under reduced pressure. Ammonium hydroxide was then added until the solution was neutral to Congo red. A precipitate of phenyl-*l*-cysteine in the form of glistening plates was obtained. The precipitate was recrystallized from hot water, filtered, dried, and washed with a small amount of ether. After drying *in vacuo*, 1.65 gm. of phenyl-*l*-cysteine were obtained which decomposed at 170–172° and gave the following results when analyzed.<sup>1</sup>

$\text{C}_9\text{H}_{11}\text{O}_2\text{NS}$ . Calculated. C 54.80, H 5.62, N 7.10  
Found. " 55.09, " 5.57, " 7.05  
 $[\alpha]_D^{25} = +11^\circ$  (1% solution in 0.1 *N* sodium hydroxide)

*Method II*—Phenyl-*l*-cysteine was also prepared by the debromination of *p*-bromophenyl-*l*-cysteine with sodium amalgam. The *p*-bromophenyl-*l*-cysteine was prepared by the method of du Vigneaud, Wood, and Binkley (9). It melted<sup>2</sup> at 193° and had a specific rotation of  $[\alpha]_D^{25} = +20^\circ$  for a 1 per cent solution in 0.1 *N* sodium hydroxide. 0.450 gm. of this material was dissolved in 10 ml. of 0.1 *N* sodium hydroxide and the solution was allowed

<sup>1</sup> All microanalyses reported in this paper were carried out by Mr. Michael Edson.

<sup>2</sup> All melting points reported herein are uncorrected.

to stand over sodium amalgam for 24 hours at room temperature. The supernatant liquid was then filtered and neutralized to Congo red by the addition of dilute sulfuric acid. A glistening white precipitate formed which was collected, washed with cold water, and dried *in vacuo*. 0.140 gm. of phenyl-*l*-cysteine was obtained which decomposed at 170–172°.

$C_9H_{11}O_2NS$ . Calculated. C 54.80, H 5.62, N 7.10  
Found. " 55.06, " 5.75, " 7.15  
 $[\alpha]_D^{25} = +11^\circ$  (1% solution in 0.1 N sodium hydroxide)

Baumann and Preusse (7) obtained phenylcysteine by the acid hydrolysis of phenylmercapturic acid. They stated that their product decomposed above 160°, but they did not report its optical rotation.

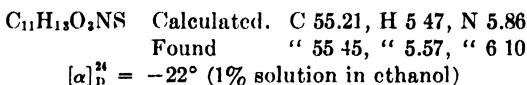
### *l*-Phenylmercapturic Acid

*Method I*—The method used by Ing, Bourne, and Young (8) for the synthesis of *l*- $\alpha$ -naphthylmercapturic acid was easily adapted to the synthesis of phenylmercapturic acid.

*l*-Cysteine was acetylated by the action of ketene according to the method of Pirie and Hele (10). The product melted at 110° and had a specific rotation of  $[\alpha]_D^{25} = +6^\circ$  for a 2.7 per cent solution in water.

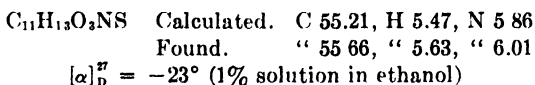
1.8 gm. of freshly distilled aniline were added to 2.5 ml. of concentrated hydrochloric acid. The precipitated hydrochloride was dissolved by adding 3 ml. of water and warming the mixture. A further 2 ml. of concentrated hydrochloric acid were added to the hot solution. The solution was then cooled quickly by the addition of 10 gm. of ice and fine crystals of aniline hydrochloride precipitated. A solution of 1.5 gm. of sodium nitrite in 5 ml. of water was then added slowly with stirring while the temperature of the mixture was kept at 0–5°. When diazotization was complete, a saturated aqueous solution of 3 gm. of sodium acetate was added to reduce the acidity of the solution. The diazo solution was then added slowly, with stirring, to 2.9 gm. of acetylcysteine dissolved in 27 ml. of water. A gummy, orange-colored product formed. The mixture was warmed gently on a water bath for 15 minutes, allowed to cool slowly, and then filtered. 3 gm. of an amorphous precipitate were obtained. 1.5 gm. of this precipitate were suspended in 25 ml. of water and 5 ml. of ethanol. Copper powder was then added and the mixture was heated on a water bath until nitrogen was no longer evolved. The mixture was then filtered and the residue was washed with boiling water. The filtrate and washings were combined, acidified by the addition of 3 ml. of concentrated hydrochloric acid, and extracted with three 50 ml. portions of chloroform. The chloroform extracts were combined and when evaporated to dryness yielded a residue of crude phenylmercapturic acid. The extracted aqueous solution

was also evaporated to dryness and from the residue a small amount of phenylmercapturic acid was obtained. A total yield of 0.180 gm. of phenylmercapturic acid which melted at 139–140° was obtained. This material was crystallized three times from hot water, dissolved in warm 60 per cent ethanol, and decolorized with charcoal. A crystalline residue of phenylmercapturic acid was obtained by evaporation of the alcoholic solution. This product was dissolved in ethanol and precipitated by the addition of water. 0.120 gm. of *l*-phenylmercapturic acid which melted at 142° was obtained.



*Method II*—Phenylmercapturic acid was prepared by the acetylation of phenyl-*l*-cysteine. The procedure used was a modification of that used by du Vigneaud, Wood, and Binkley (9) to acetylate *p*-bromophenyl-*l*-cysteine.

The phenyl-*l*-cysteine used was prepared by Method I described earlier in the present paper. It decomposed at 170–172° and had a specific rotation of  $[\alpha]_D^{25} = +11^\circ$  for a 1 per cent solution in 0.1 *N* sodium hydroxide. 0.189 gm. of this material was dissolved in 4 ml. of ice-cold 1 *N* sodium hydroxide. The solution was immersed in an ice bath and 0.25 ml. of acetic anhydride was added slowly with vigorous stirring, followed by 2 ml. of 1 *N* sodium hydroxide and another 0.25 ml. of acetic anhydride. The solution was stirred vigorously for a further 5 minutes and then made acid to Congo red by the addition of sulfuric acid. The solution became turbid and after standing for 24 hours in the refrigerator the crystals which had separated were collected and recrystallized from aqueous ethanol. 0.125 gm. of *l*-phenylmercapturic acid, melting at 142°, was obtained.



*Method III*—Baumann and Preusse (7) prepared phenylmercapturic acid, m.p. 142–143°, by the debromination of *p*-bromophenylmercapturic acid isolated from the urine of dogs which had been dosed with bromobenzene. The preparation of phenylmercapturic acid by this method has now been repeated.

The *p*-bromophenylmercapturic acid used in the experiment described below was synthesized by the method of du Vigneaud, Wood, and Binkley (9). It melted at 151–152° and had a specific rotation of  $[\alpha]_D^{25} = -13^\circ$  for a 1 per cent solution in 95 per cent ethanol. 0.400 gm. of the *p*-bromophenylmercapturic acid was dissolved in 20 ml. of 0.5 *N* sodium hydroxide and the solution was allowed to stand for 48 hours over freshly prepared

sodium amalgam. The supernatant solution was then filtered, made acid to Congo red, and allowed to stand overnight in the refrigerator. 0.210 gm. of *l*-phenylmercapturic acid was obtained which melted at 142.5°.

$C_{11}H_{13}O_2NS$ . Calculated, C 55.21, H 5.47; found, C 55.40, H 5.70  
 $[\alpha]_D^{25} = -23^\circ$  (1% solution in ethanol)

#### SUMMARY

1. Phenyl-*l*-cysteine has been prepared by two methods: (a) by decomposing the product of the interaction of diazotized aniline and cuprous cysteine mercaptide, and (b) by the debromination of *p*-bromophenyl-*l*-cysteine with sodium amalgam.

2. *l*-Phenylmercapturic acid has been prepared by three methods: (a) by decomposing the product of the interaction of diazotized aniline and acetyl-cysteine, (b) by the acetylation of phenyl-*l*-cysteine, and (c) by the debromination of *p*-bromophenylmercapturic acid with sodium amalgam.

One of us (S. H. Z.) is indebted to the Banting Research Foundation for a personal grant.

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# MERCAPTURIC ACIDS

## II. THE FORMATION OF *l*-PHENYLMERCAPTURIC ACID FROM PHENYL-*l*-CYSTEINE IN VIVO

By S. H. ZBARSKY AND LESLIE YOUNG

(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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The formation of phenylmercapturic acid *in vivo* has not been demonstrated hitherto, although indications that this compound is excreted by animals after the administration of benzene have been reported (1-3). It has been shown by Stekol (4) and by du Vigneaud, Wood, and Binkley (5) that *p*-bromophenyl-*l*-cysteine is converted to *p*-bromophenylmercapturic acid by the rat and that the latter compound is excreted in the urine. It therefore appeared that phenyl-*l*-cysteine in a similar manner might undergo conversion to phenylmercapturic acid in the organism of the rat. The occurrence of this change has now been established by the isolation of *l*-phenylmercapturic acid from the urine of the rat following the ingestion of phenyl-*l*-cysteine.

### EXPERIMENTAL

The procedure by which phenylmercapturic acid was isolated from rat urine was based on that used by Bourne and Young (6) for the isolation of *l*- $\alpha$ -naphthylmercapturic acid from the urine of rabbits to which naphthalene had been administered. The urine was made acid to Congo red by the addition of hydrochloric acid and after several hours it was extracted with chloroform. The chloroform extract was evaporated to dryness and the residue was crystallized from aqueous ethanol. The efficiency of this procedure was tested in a series of experiments in which it was applied to samples of normal rat urine containing added phenylmercapturic acid. These experiments showed that it was possible to isolate phenylmercapturic acid from rat urine when as little as 0.012 gm. of the compound was present in 50 ml. of urine.

Three separate experiments were performed in which *l*-phenylmercapturic acid was isolated from the urine of rats which had ingested phenyl-*l*-cysteine. One of these experiments is described in detail below.

A male white rat weighing 240 gm. was fed the stock colony diet<sup>1</sup> to which had been added 2 per cent by weight of phenyl-*l*-cysteine. The phenyl-*l*-cysteine used was synthesized by Method I described in the preceding

<sup>1</sup> Ground corn 50, dried milk 25, linseed meal 15, casein 3.5, alfalfa meal 1.5, calcium carbonate 0.5, sodium chloride 0.5, yeast powder 2, and cod liver oil 2 parts.



paper (7). It decomposed at 170–172° and had a specific rotation of  $[\alpha]_D^{25} = +11^\circ$  for a 1 per cent solution in 0.1 N sodium hydroxide. In order to avoid contamination of the urine with fallen food, the rat was fed in a separate cage twice a day (morning and early evening) for periods each lasting 1 hour and the urine excreted during these periods was discarded. The animal had access to drinking water at all times. Feeding of the diet containing phenyl-*l*-cysteine was continued for 3 days and during this period the rat ingested 0.6 gm. of the compound. The urine was collected during this period and for 2 days afterward. 34 ml. of urine were collected. This was acidified to Congo red by the addition of concentrated hydrochloric acid and centrifuged immediately to remove a feathery precipitate which formed. Examination of this precipitate failed to reveal the presence of phenylmercapturic acid. The supernatant liquid was allowed to stand in the refrigerator for 24 hours and was then extracted by shaking with three 50 ml. portions of chloroform. The emulsions which formed were broken by centrifuging. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the chloroform was removed by evaporation. The residue was dissolved in a few ml. of ethanol. Dilution of this solution with water resulted in the formation of crystals of impure phenylmercapturic acid. The material weighed 0.228 gm. and melted at 138–139°. It was dissolved in hot aqueous ethanol and the solution was decolorized with charcoal. The filtered solution was concentrated and yielded 0.219 gm. of crystalline *l*-phenylmercapturic acid which melted at 141–142°. The melting point was not depressed when the isolated compound was mixed with synthetic *l*-phenylmercapturic acid, m.p. 142°, prepared by Method II described in the preceding paper. Analysis<sup>3</sup> of the isolated compound yielded the following results.

$C_{11}H_{13}O_2NS$	Calculated.	C 55.21, H 5.47, N 5.86
	Found.	" 55.31, " 5.53, " 6.18
$[\alpha]_D^{25}$		$-22^\circ$ (1% solution in ethanol)

In a similar experiment to that described above a male white rat ingested 0.250 gm. of phenyl-*l*-cysteine and from the urine 0.088 gm. of *l*-phenylmercapturic acid, m.p. 142°, was isolated. In another experiment a male white rat ingested 0.600 gm. of phenyl-*l*-cysteine and 0.275 gm. of *l*-phenylmercapturic acid, m.p. 142°, was isolated from the urine. In the above experiments the amounts of phenylmercapturic acid isolated from the urines were equivalent to 30, 29, and 38 per cent respectively of the phenyl-*l*-cysteine ingested.

<sup>2</sup> All melting points reported herein are uncorrected.

<sup>3</sup> Microanalyses by Mr. Michael Edson.

## SUMMARY

*l*-Phenylmercapturic acid has been isolated from the urine of rats following the administration of phenyl-*l*-cysteine in the diet.

One of us (S. H. Z.) is indebted to the Banting Research Foundation for a personal grant.

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# FURTHER STUDIES ON ISOLATED CELL NUCLEI OF NORMAL RAT LIVER

By ALEXANDER L. DOUNCE

*(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry,  
The University of Rochester, Rochester, New York)*

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A method for preparing nuclei of the cells of normal rat liver at pH 6.0 to 6.2 already has been published (1) together with an account of six important enzymes found to be present in these nuclei in relatively high concentration. The principal objects of the present paper are twofold. The first is to compare nuclei isolated from cells of normal rat liver at pH 6.0 to 6.2 (1) with nuclei isolated at a lower pH range of 3.8 to 4.0, in regard to total lipid content and desoxyribonucleic acid content.

The second object of the paper is to show that, in isolated cell nuclei prepared by certain methods, the nucleic acid can exist either in a loosely combined, easily extractable state, or that, in nuclei prepared under different conditions, it may exist in a more firmly bound, largely unextractable state relative to mild extraction procedures. This already has been reported briefly (2). As has been stated previously, purified nucleic acid obtained by a mild extraction procedure from nuclei of rat liver cells prepared at pH 6.0 to 6.2 is in a polymerized state.

The purpose of comparing nuclei isolated at pH 6.0 to 6.2 with nuclei isolated at pH 3.8 to 4.0 is to attempt to establish whether appreciable material has been extracted from the nuclei prepared at the higher pH range during the procedure for their isolation. For reasons which will be taken up in the discussion, we believe that all of the major constituents of high molecular weight of nuclei prepared at pH 3.8 to 4.0 are retained by the nuclei during the process of isolation, although protein of high molecular weight is largely denatured. These constituents of high molecular weight comprise nucleohistone, ordinary protein, and lipid.

If ordinary protein should be extracted from nuclei prepared at pH 6.0 to 6.2, it would follow that at least some of the enzymes also would be partially extracted, resulting in a lowered enzyme activity of the nuclei. It will be shown that in all probability some protein may in fact be extracted from nuclei prepared at the higher pH range, and that therefore the measured enzyme concentrations of nuclei isolated at this pH range may be somewhat lower than the true concentrations of the enzymes as they exist in the living cell.

During the course of the work on nucleic acid, it was found desirable to

compare fractions containing nucleic acid and protein extracted from nuclei prepared at pH 6.0 to 6.2 with corresponding fractions obtained from nuclei prepared at pH 3.8 to 4.0, particularly in regard to absorption spectra. Histone fractions also were prepared from nuclei isolated at pH 6.0 to 6.2 and at pH 3.8 to 4.0, and the absorption spectrum of one of these fractions in the ultraviolet region was determined. All of the absorption spectra included in this paper, as well as many others not included, were determined by Dr. L. T. Steadman of the Department of Radiology.

In regard to extractable nucleoprotein fractions prepared from the nuclei, it should be stated that these fractions in all probability consist of nucleic acid combined loosely with histone, together with varying amounts of ordinary protein which is not combined with the nucleic acid. According to the most recent work published (3, 4), purified nucleoprotein from cell nuclei consists of nucleohistone. The nucleoprotein of nuclei thus is distinctly different in type from the firmly bound protein-ribose nucleic acid combinations which comprise some of the isolated viruses (5, 6) and the fine cytoplasmic granules of animal cells (7).

In addition to experimental work bearing on the two chief objects of the paper, some additional experiments are reported concerning a search for glycogen and a further search for cytochrome *c* in nuclei of rat liver cells.

#### EXPERIMENTAL

*Preparation of Cell Nuclei of Rat Liver at pH 3.8 to 4.0*—A mixture of roughly equal parts of crushed ice and distilled water containing 20 cc. of molar citric acid is made up to a volume of 500 cc. and is placed in a Waring blender or other suitable high speed mixer. 100 gm. of frozen rat liver are now added in small pieces as rapidly as possible without stalling the blender. The blender is run until the ice has melted, care being taken to break up at once any mass of crushed ice which forms at the top of the suspension while it is being mixed. When the ice has all melted, the mixer is stopped and the nuclei are isolated in the way already described for the preparation of cell nuclei of rat liver at pH 6.0 to 6.2 (1). There is one minor point of deviation from the procedure for nuclei isolated at pH 6.0 to 6.2, however. After the nuclei liberated at pH 3.8 to 4.0 have been washed once or twice, the pH tends to rise above 4.0 and the nuclei then agglutinate, making further washing useless. To prevent this agglutination, the pH should be maintained at 3.8 to 4.0 by the addition of a few drops of molar citric acid as needed. There will be very little trouble with loosely packed sediment, and for this reason the nuclei prepared at pH 3.8 to 4.0 may require less washing than nuclei prepared at pH 6.0 to 6.2. Fig. 1 shows an average preparation of these nuclei.

*Preparation of Cell Nuclei of Rat Liver at pH 6.0 to 6.2*—The method

employed has already been described, and a photograph of these nuclei has been published (1).

*Preparation of Nuclei of Chicken Erythrocytes*—The method of Dounce and Lan (8) was employed, saponin being used to lase the cells in 0.85 per cent sodium chloride solution. A photograph of these nuclei will appear elsewhere.

*Analysis of Isolated Nuclei for Total Desoxyribonucleic Acid*—The nuclei were well suspended in water and an aliquot of 0.5 or 1.0 cc. of the suspension was dried in the oven at 100° for the determination of dry weight. For the determination of nucleic acid, the Dische reaction (9) as employed by Seibert (10) was used. The reagent is prepared by dissolving 2.75 gm. of concentrated sulfuric acid and 1.0 gm. of diphenylamine in 100 cc. of glacial acetic acid. The blank reagent is made by dissolving 2.75 gm. of

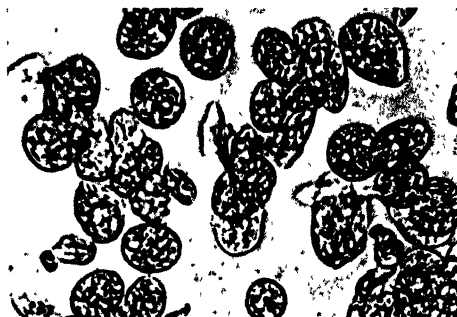


Fig 1 Nuclei isolated from rat liver cells (Wistar) at pH 3.8 to 4.0.  $\times 720$

sulfuric acid in 100 cc. of glacial acetic acid, but the diphenylamine is omitted.

0.5 cc. of the suspension of nuclei was added to each of two test-tubes. To one of the test-tubes 1.0 cc. of the Dische reagent was then added and to the other 1.0 cc. of the blank reagent was added. The tubes were immediately immersed in boiling water and were left there for 10 minutes. They were then cooled under tap water and to the tube containing the Dische reagent 8.5 cc. of the blank reagent were added. To the tube containing the blank reagent were added 1.0 cc. of the Dische reagent and 7.5 cc. of the blank reagent, so that the total volume of liquid in both tubes was now 10 cc. The contents of both test-tubes were transferred to centrifuge tubes and were centrifuged for 10 minutes at high speed to remove a precipitate which formed upon dilution with the blank reagent. After centrifuging, the supernatant solutions were read in the photoelectric colorimeter at once, and the blank was subtracted from the unknown. The total length of time elapsing between the time the tubes are placed

in the centrifuge and the time at which the colorimeter readings are taken should be kept constant and as short as possible, since the color increases on standing.

Fig. 2 shows a curve made by plotting the concentration of sodium desoxyribonucleate prepared according to Hammarsten (11) against the corresponding colorimeter readings from which the blank readings have been subtracted. The concentration of nucleic acid may be obtained by multiplying the concentration of the sodium salt by 0.944.

After the concentration of an unknown has been determined by means of this curve, if one wishes to improve the accuracy, the determination may be repeated, this time running together with the unknown a standard solution of the same strength as has been calculated for the strength of the

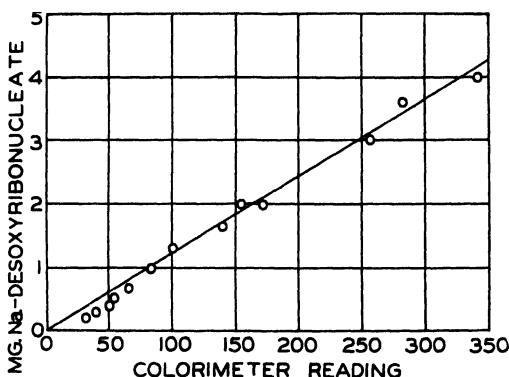


FIG 2 Curve showing the mg of sodium desoxyribonucleate (ordinate) plotted against the colorimeter reading (abscissa), the blank being subtracted Klett-Summerson photoelectric colorimeter, No 54 filter The sodium desoxyribonucleate used in plotting the curve was about 86 per cent pure The curve is not corrected for this impurity.

unknown by use of the curve. Since exactly the same conditions are applied to both unknown and standard, any error caused by slight differences in conditions should be compensated. The concentration of the unknown is now calculated again, the following equation being used.

$$\text{Concentration of unknown} = \frac{\text{concentration of standard}}{\text{reading for standard}} \times \text{reading for unknown}$$

Usually this value is not far removed from the first value obtained by using the standard curve.

Spectroscopic analysis of our standard sodium desoxyribonucleate prepared according to the method of Hammarsten indicated the presence of some impurity. For this reason, phosphorus analyses were made<sup>1</sup> and

<sup>1</sup> We are indebted to Dr R. F. Riley of this department for carrying out the phosphorus determinations.

the percentage of phosphorus found was compared with the percentage of phosphorus calculated for a tetranucleotide molecule containing two purine and two pyrimidine components. The results of the analysis gave an average value of 8.0 per cent for phosphorus, while the theoretical value is 9.25 per cent. This indicated that the standard was about 86 per cent pure, and hence all figures for nucleic acid were multiplied by 0.86 to correct for the impurity of the standard.

*Analysis of Nuclei for Total Lipid*—The total lipid was extracted from samples of dried nuclei in a small Soxhlet extractor with a solvent mixture consisting of 25 per cent ether and 75 per cent alcohol. The solvent was evaporated and the lipid residue was determined by direct weighing, since preliminary experiments had demonstrated that it was entirely soluble in petroleum ether and therefore could not have contained appreciable non-lipid material. Further extraction of the nuclei with a chloroform-methanol mixture did not remove any more lipid.

The samples of nuclei for total lipid analysis were dried either by lyophilizing or by heating in an oven at 100°. Both methods appeared to be satisfactory. The nuclei of chicken erythrocytes were first precipitated by adding dilute acetic acid until the pH was about 3.5, and then were washed at this pH with very dilute acetic acid to remove sodium chloride before being dried.

*Procedure for Extracting Nucleic Acid and Protein from Cell Nuclei of Rat Liver Prepared at pH 3.8 to 4.0 or at pH 6.0 to 6.2*—The water suspension of nuclei is centrifuged and the nuclei are washed three or four times with Ringer's phosphate solution at pH 7.4. By this procedure little if any nucleic acid is removed, but considerable protein is extracted from nuclei prepared at pH 6.0 to 6.2. From nuclei prepared at pH 3.8 to 4.0, only a very small amount of protein is removed, probably because the protein previously has been denatured at the low pH employed in the preparation of these nuclei.

Next the nuclei are extracted two or three times with small portions of 5 per cent sodium chloride solution. The centrifuge with high speed attachment must be used to centrifuge down the residue from nuclei prepared at pH 6.0 to 6.2, at a speed of 12,000 to 15,000 R.P.M.

The combined 5 per cent sodium chloride extracts from nuclei prepared at pH 6.0 to 6.2 contain practically all of the nucleic acid, together with protein. From nuclei prepared at pH 3.8 to 4.0, only a fraction of the nucleic acid is extracted, which is in the neighborhood of 10 per cent of the total amount present.

The ultraviolet absorption spectrum of the "nucleoprotein" fraction extracted with 5 per cent sodium chloride solution from cell nuclei of rat liver prepared at pH 6.0 to 6.2 is shown in Fig. 3. Analysis of this fraction for desoxyribonucleic acid gave a value of about 17 per cent nucleic acid



nitrogen based on total nitrogen. The spectrum of the corresponding fraction obtained from the nuclei prepared at pH 3.8 to 4.0 also is shown in Fig. 3. Analysis of this fraction gave a value for deoxyribonucleic acid nitrogen of about 10 per cent based on total nitrogen. Total nitrogen was determined by the Kjeldahl procedure, and the nucleic acid nitrogen was calculated from total nucleic acid determined by the Dische reaction.

Following the extraction with sodium chloride, a very small amount of protein and nucleic acid can be removed from the residue from nuclei prepared at either pH 3.8 to 4.0 or pH 6.0 to 6.2 by washing once or twice with 0.1 M phosphate buffer of pH 7.5 to 7.6. The residue from nucle.

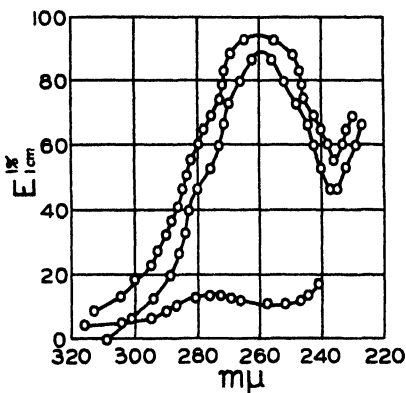


FIG. 3. The top curve represents the absorption spectrum of the nucleoprotein fraction soluble in 5 per cent sodium chloride from cell nuclei of rat liver isolated at pH 6.0 to 6.2; the middle curve, the absorption spectrum of the nucleoprotein fraction soluble in 5 per cent sodium chloride from cell nuclei of rat liver isolated at pH 3.8 to 4.0; the bottom curve, the absorption spectrum of the histone fraction at pH 4.0 prepared from cell nuclei of rat liver isolated at pH 6.0 to 6.2.  $E_{1\text{cm}}^{1\%} = (\log_{10} I_0/I) \times 1/c \times 1/d$ , where  $c$  is the concentration in per cent and  $d$  is the path length in cm.

prepared at pH 6.0 to 6.2 must be centrifuged in the centrifuge with high speed attachment, as before.

The final residue obtained after washing with phosphate buffer of pH 7.4 in the case of nuclei prepared at pH 6.0 to 6.2 contains protein and lipid. Apparently it consists of nuclear membranes together with insoluble material from the interior of the nuclei.

The final residue obtained from nuclei prepared at pH 3.8 to 4.0 contains protein, lipid, and nucleic acid. If this residue is washed two or three times with distilled water, and then if distilled water is added and the pH is adjusted to 8.0 to 8.5 by the addition of ammonia, a gel is formed which persists in high dilution. A similar gel is formed by treating the original nuclei prepared at pH 3.8 to 4.0 with dilute ammonia at pH 8.0 to 8.5.

Neither the residue from nuclei prepared at pH 6.0 to 6.2 nor the nuclei themselves will form such a gel. Since the nuclei prepared at pH 3.8 to 4.0 retain much of their nucleic acid in a firmly bound condition up to the point at which the gel is formed, it appears likely that the presence of firmly bound nucleic acid is necessary for the formation of the gel. An apparently similar gel formed by adding ammonia to extracted liver tissue has been reported by Bensley and Hoerr (12).

*Alternate Procedure for Removal of Protein and Soluble Nucleic Acid from Nuclei Prepared at pH 3.8 to 4.0*—One adjusts the pH of a moderately concentrated suspension of cell nuclei of rat liver, prepared at pH 3.8 to 4.0, to pH 7.5 by the addition of a small amount of disodium phosphate or sodium bicarbonate solution. The nuclei swell and a certain amount of protein is dissolved. The nuclei are centrifuged and are extracted two or three times more at pH 7.5 to 7.6 with 0.5 per cent sodium chloride solution. The addition of the sodium chloride controls the swelling of the nuclei. The combined extracts are brought to pH 4.0 to 4.5 by the addition of molar acetic acid, and the precipitate, which consists of nucleic acid and protein, is centrifuged. This precipitate is entirely soluble in dilute disodium phosphate or sodium bicarbonate at pH 7.5 to 7.6, but it is only partially soluble in 5 or 10 per cent sodium chloride solution.

Most of the nucleic acid, together with some protein, can be extracted from this precipitate with 5 or 10 per cent sodium chloride solution. The material insoluble in sodium chloride contains a very small amount of nucleoprotein soluble in dilute sodium carbonate or phosphate at pH 7.5, and a fraction that is soluble at pH 2 to 3, which may be histone. The remainder of the precipitate is insoluble under mild conditions and appears to consist of denatured protein.

The residue of somewhat swollen nuclei, after being washed once or twice with distilled water, gives a gel at pH 8.0 to 8.5 in the presence of ammonia, which contains protein, lipid, and nucleic acid, and which is similar to or identical with the gel already described.

This alternate procedure for the separation of protein fractions has been applied to cell nuclei of Walker Carcinosarcoma 256, prepared at pH 3.0 with 1.5 per cent citric acid, but the results were not different from those described for nuclei of cells of normal rat liver.

*Preparation of Histone Fraction from Isolated Cell Nuclei of Rat Liver*—Nuclei isolated either at pH 6.0 to 6.2 or at pH 3.8 to 4.0 may be used in preparing this fraction. The nuclei are lyophilized and then are extracted two or three times with small volumes of 0.1 N HCl. The residue is removed by filtration or centrifugation, and the combined filtrates or supernatants are adjusted to a pH value near 5.0. Then 3 volumes of alcohol

are added and the material is placed in the ice box until the precipitated histone flocculates. The flocculated precipitate then is centrifuged down and is dissolved by adding a small amount of water and just sufficient 0.1 N HCl to cause the precipitate to pass into solution. Any trace of precipitate that remains undissolved is removed by high speed centrifugation. The histone may be reprecipitated by alcohol if desired.

The histone fraction thus prepared is soluble in dilute HCl, but if the pH is raised sufficiently by the addition of 0.1 N NaOH, it will precipitate. Near pH 8.0 it is still soluble, although slightly opalescent. The material is easily salted-out with sodium chloride.

The absorption spectrum at pH 4.0 of the histone fraction prepared from nuclei isolated at pH 6.0 to 6.2 is shown in Fig. 3.

*Search for Glycogen in Isolated Nuclei*—Two preparations of nuclei of cells of normal rat liver prepared at pH 3.8 to 4.0 and one preparation isolated at pH 6.0 were examined for glycogen content. The nuclei were centrifuged down from a water suspension and then were made into a paste by the addition of a small amount of distilled water. Sand was added to this paste and the mixture was ground in a mortar until microscopic examination showed that the nuclei had been reduced to fine particles. A 5 per cent trichloroacetic acid solution was then used to extract the ground nuclei, care being taken to use small amounts, so that a concentrated extract would be obtained. It was originally intended to make quantitative determinations of the glycogen precipitable by alcohol from the trichloroacetic acid extract, but, upon addition of 2 volumes of 95 per cent alcohol to the extract, no visible precipitate appeared, so that it was impossible to proceed further. All three of the preparations of nuclei gave the same negative results.

It is improbable that a material with as high a molecular weight as glycogen should be entirely extracted from the nuclei during their isolation, while much or most of the ordinary protein should remain, as it does. It might be argued that enzyme action would rapidly destroy the glycogen in nuclei isolated at pH 6.0, but this would not be likely to occur in nuclei isolated at pH 3.8 to 4.0. Furthermore, very fresh preparations of nuclei were used, and in each case glycogen was readily isolated from the original cytoplasmic fractions after the nuclei were centrifuged down.

Therefore we have concluded that glycogen is very low or absent in the cell nuclei of normal rat liver.

*Further Search for Cytochrome c in Nuclei Isolated from Rat Liver Cells at pH 6.0 to 6.2*—In the previous paper on isolated cell nuclei of rat liver (1), it was stated that cytochrome *c* was absent from these nuclei, since spectroscopic evidence for its presence was not found, and since it was necessary to add cytochrome *c* to the nuclei in order to obtain appreciable

cytochrome oxidase activity. Recently we have found that, if fresh preparations of cell nuclei of rat liver are extracted with a small amount of 5 per cent NaCl buffered to about pH 7.5 with phosphate, it is possible to demonstrate a small concentration of cytochrome *c* in the filtered extract. A very small quantity of solid  $\text{Na}_2\text{S}_2\text{O}_4$  was added to such an extract prepared from fresh nuclei, and the solution was examined spectroscopically with a small pocket spectroscope having a scale. The small instrument was used to obtain a concentrated spectrum. The  $\alpha$ -line of cytochrome *c* could be seen superimposed on the side of the faint hemoglobin band which is toward the violet end of the spectrum. The  $\beta$ -band was barely visible.

In order to identify the bands, a known sample of cytochrome *c* was diluted until the bands were approximately of the same intensity as those produced by the extract of nuclei, and then the location of both sets of bands on the scale of the instrument was observed. Corresponding bands of the known cytochrome *c* sample and of the extract of nuclei fell on exactly the same scale readings.

Therefore we wish to correct the previous statement that cytochrome *c* is absent from nuclei isolated from the cells of normal rat liver at pH 6.0 to 6.2, and to state instead that it is present but in low concentration. The principal reason for our failure to detect the cytochrome *c* before was that we did not add  $\text{Na}_2\text{S}_2\text{O}_4$  to obtain the spectrum of reduced cytochrome *c* which is much easier to detect than that of the oxidized material. Cytochrome *c* in the isolated nuclei is in the oxidized state. The concentration of this substance in the nuclei appears to be so low that it would be difficult to decide whether it actually belongs there or is merely adsorbed during the preparation of the nuclei, as hemoglobin appears to be (1).

*Attempted Fractionation of Nuclei of Bird Erythrocytes*—All attempts to prepare fractions of soluble nucleoprotein from nuclei of bird erythrocytes so far have failed. The nuclei form a gel in 5 or 10 per cent sodium chloride solution, saturated sodium chloride (13), or in alkaline buffer. In distilled water, they agglutinate and form a single mass of material which gives a gel on attempts to extract it in neutral or alkaline solutions. It seems likely that anything which completely destroys the tenuous stroma, stable in 0.85 per cent sodium chloride solution at pH 7.0, will favor agglutination if the nuclei are agitated or centrifuged.

It is not likely that appreciable protein is extracted from the nuclei of chicken erythrocytes by the 0.85 per cent sodium chloride solution used in preparing them, since the second washing of the nuclei with this solution is almost protein-free. Moreover, the nuclei do not appear to shrink during treatment with 0.85 per cent saline, as do nuclei of rat liver cells when they are treated with Ringer's phosphate solution, which extracts protein from them.

## DISCUSSION

It appears probable that little material of high molecular weight is lost from the nuclei isolated at pH 3.8 to 4.0. Protein of high molecular weight is undoubtedly denatured and thereby rendered insoluble; and nucleohistone must be quite insoluble at this pH range, which is very close to pH values usually reported for isoelectric points of nucleohistone (14). There is no reason why appreciable lipid should be removed from intact nuclei prepared at pH 3.8 to 4.0. Glycogen has been shown to be low or absent in nuclei of rat liver cells. Therefore it seems reasonable to use

TABLE I

*Percentage of Total Lipid and Desoxyribonucleic Acid in Cell Nuclei of Rat Liver*

Each figure for desoxyribonucleic acid represents an average of two or more determinations on a given preparation of nuclei, and each figure for total lipid represents a single determination on a given preparation of nuclei. From ten to fifteen rat livers were used in making a given preparation of nuclei.

	Nuclei of normal rat liver	
	pH 3.8-4.0	pH 6.0-6.2
Desoxyribonucleic acid	22.6*	12.8*
	20.2*	20.2*
	23.9*	18.8†
	22.4†	16.2†
	19.7†	
Lipid	3.2*	7.5*
	3.2*	10.7††
	6.0†	10.8††
	6.0†	
	6.3†	
	7.2†	

\* Rats of the Osborne-Mendel strain were used.

† Rats of the Wistar strain were used.

†† Values previously reported (1).

the analytical values for lipid and desoxyribonucleic acid obtained for nuclei prepared at pH 3.8 to 4.0 as standard values with which to compare the corresponding values found for nuclei prepared at other pH values or by other methods.

Nuclei prepared at pH values much below 3.0 undoubtedly lose much or all of their histone content, as will be demonstrated elsewhere, and so will give high values for lipid and nucleic acid. On the other hand, as may be seen from the figures in Table I, nuclei isolated at pH 6.0 to 6.2 appear to lose some nucleic acid, and therefore give somewhat high values for total lipid content. The amount of nucleic acid lost appears to be somewhat variable, as can be seen from Table I, and preliminary observations not

included in the table indicate that the nuclei which have lost the greatest amount of nucleic acid show the highest values for total lipid. It appears probable that nuclei stirred in the Waring blender so long that they become broken are those which lose relatively large amounts of nucleic acid.

It seems unlikely that nuclei should lose nucleic acid without also losing some protein, and therefore some of their enzyme content. For this reason it is possible that some of the values reported for the concentrations of certain enzymes of the isolated nuclei (1) may actually be lower than the true concentrations of these enzymes in the nuclei as they exist in the living cell. However we do not wish to apply this reasoning to the enzymes catalase and succinic dehydrogenase, which were found to be absent or present only in traces. There seems to be no reason why these enzymes should be almost quantitatively removed from nuclei, while the other enzymes investigated should remain in relatively high concentrations. These two enzymes probably are lacking in the nuclei as they exist in the living cell.

In connection with the lipid analyses, it is of interest to note that the total lipid content of nuclei of liver cells of rats of the Wistar strain appears to be higher than the total lipid of nuclei of liver cells of Osborne-Mendel rats.

In regard to the state of the nucleic acid in isolated nuclei, it has been shown that this substance can exist either in an easily extractable, loosely combined state, or that it can be to a considerable extent in a more firmly bound, non-extractable state, presumably combined with protein. Either state can exist in nuclei which have been prepared by relatively mild methods. Thus in nuclei of rat liver cells prepared at pH 6.0 to 6.2, we have the nucleic acid almost entirely in the loosely combined state, while in nuclei of rat liver cells prepared at pH 3.8 to 4.0, as well as in nuclei of chicken erythrocytes prepared at pH 6.8 to 7.0, the nucleic acid is largely in the firmly combined state. It seems obvious that the nucleic acid of nuclei must exist in the loosely combined state or be thrown into this state when tissues are subjected to procedures such as that of Hammarsten (11) for preparing thymus nucleic acid, or of Mirsky and Pollister (3, 4) for preparing nucleoproteins. Otherwise, the yields of nucleic acid or nucleoprotein would be very small. Under the same conditions, the nucleic acid of animal cell cytoplasm, which is known to exist in granules as a liponucleoprotein complex (7), must remain firmly combined, or otherwise it would contaminate the desoxyribonucleic acid extracted from the nuclei.

Denaturation of protein evidently is not responsible for the firmly bound state of the nucleic acid of isolated nuclei, since this state occurs in nuclei of chicken erythrocytes which are prepared at too high a pH for denaturation of protein, and since in general denaturation is stated to liberate firmly

bound nucleic acid from protein (5, 6, 15). It might be thought that when nuclei are prepared at pH 3.8 to 4.0 a change in the permeability of the nuclear membrane could cause the nucleic acid to become unextractable. However, this is not probable in view of the fact that a small amount of the nucleic acid actually can be extracted from these nuclei. Moreover, the nuclei of chicken erythrocytes, which have all of their nucleic acid in a firmly combined state, were not prepared at low pH. Finally, when nuclei of liver cells prepared at pH 3.8 to 4.0 are suspended in 100 cc. of distilled water and then are fragmented by prolonged stirring at high speed in the Waring blender, the nucleic acid still is firmly bound to the residue obtained by centrifugation at high speed. This was determined by extracting the residue with 5 and then 10 per cent solutions of sodium chloride and analyzing the residue for nucleic acid. The nucleic acid content was found to be 19.5 per cent, and very little precipitable nucleoprotein appeared in the extract.

It is interesting to speculate as to whether the two states of nucleic acid in nuclei are physiologically reversible, or whether one state is normal for the living cell and the second state occurs after death or disruption of the cell, but at present these questions do not seem to be answerable from biochemical evidence. Changes in the viscosity of nuclei of tumor cells have been reported (16), and possibly these changes might be connected with a change in the state of combination of the nucleic acid in the nuclei.

#### SUMMARY

1. A method has been given for isolating cell nuclei of rat liver at pH 3.8 to 4.0. This pH results in denaturation of much protein, but is favorable to retention of nucleic acid and protein by the nuclei.

2. A study has been made of the total lipid and desoxyribonucleic acid content of cell nuclei of rat liver prepared at pH 3.8 to 4.0 and at pH 6.0 to 6.2. From this it is concluded that some protein is probably lost from nuclei prepared at the higher pH range, and that therefore the true concentrations of several enzymes already reported to be present in the nuclei may be even higher than the reported values.

3. It has been shown that the desoxyribonucleic acid of isolated cell nuclei may exist either in an easily extractable, loosely combined state, or in a more firmly combined state, unextractable by mild procedures. Nuclei of bird erythrocytes have been included in this study.

4. Some absorption spectra have been given for the principal nucleic acid-containing fractions prepared from isolated nuclei of rat liver cells.

5. Glycogen appears to be very low or absent in nuclei of rat liver cells.

6. Cytochrome *c* appears to be present in low concentration in nuclei isolated from cells of normal rat liver, instead of being absent as was previously reported.

We wish to express our gratitude to The International Cancer Research Foundation of Philadelphia, Pennsylvania, whose financial support has made this work possible.

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# THE DESOXYRIBONUCLEIC ACID CONTENT OF ISOLATED NUCLEI OF TUMOR CELLS

By ALEXANDER L. DOUNCE

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry,  
The University of Rochester, Rochester, New York)

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In a recent communication Koller (1) has stated his belief that the nucleic acid content of chromosomes rises to abnormally high values in tumor cells. Apparently he believes that this increased content of nucleic acid is related to the cause of malignancy, as he makes the following statement. "Since the amount of nucleic acid and the rate of its production determine the frequency of division, the excess amount of nucleic acid present in the tumour must be considered as the fundamental cause of increased division rate and malignancy."

In this paper we should like to report the results of analyses for desoxyribonucleic acid in nuclei isolated from the cells of the two types of rat tumor, and to compare these results with the total desoxyribonucleic acid content of cell nuclei of normal rat liver. Also, in order to show that other types of cell nuclei may have a much higher desoxyribonucleic acid content than either the cell nuclei of normal liver or the nuclei of the two tumor cells studied, we have included analyses for the desoxyribonucleic acid content of the nuclei of fish spermatozoa and bird erythrocytes.

## EXPERIMENTAL

*Preparation of Cell Nuclei of Normal Rat Liver*—These nuclei were prepared at pH 3.8 to 4.0 according to the method given in the preceding paper from the livers of rats of the Osborne-Mendel and Wistar strains.

*Preparation of Cell Nuclei of Walker Carcinoma 256*—The tumors were frozen and the necrotic areas were discarded. Then 100 gm. of frozen tumor were added rapidly to a Waring blender containing 500 cc. of a mixture of crushed ice, distilled water, and 37.6 cc. of molar citric acid. The blender was allowed to run for 15 minutes. The final pH of the mixture was about 3.0. Before the tumor was added, the concentration of the citric acid in the blender was about 1.5 per cent.<sup>1</sup> The nuclei were then strained through cheese-cloth and were isolated by repeated differential centrifugation in an ordinary centrifuge. After the first washing, a drop or two of molar citric acid was added when needed to prevent agglutination of the nuclei because of increase in pH.

<sup>1</sup> Nuclei of Walker Carcinoma 256 have been isolated by Haven and Levy (2) by the use of 2 per cent citric acid.

The use of pH 3.0 instead of pH 3.8 to 4.0 undoubtedly results in the removal of a certain amount of histone from the nuclei, since pH 3.0 probably is near the critical value below which the histone begins to be dissociated from the nucleic acid and since nuclei of liver cells prepared at pH 3.0 appear to lose some histone. According to the directions in Kossel's book (3) for preparing thymus histone from thymus nucleohistone, the solution must be definitely acid to Congo red in order that splitting of the histone from the nuclei may take place. This would imply a pH range of 2 to 3. It would be desirable to prepare cell nuclei of Walker tumors at pH 3.8 to 4.0, but so far this has proved to be impossible, since the cytoplasm is not well removed from the nuclei at this pH range.

A photograph of nuclei isolated from Walker Carcinosarcoma 256 at pH 3.0 is shown in Fig 1.



FIG 1 Nuclei isolated at pH 3.0 from Walker rat Carcinosarcoma 256  $\times 720$

*Preparation of Isolated Nuclei from Hepatoma 31*—It was found impossible to obtain good preparations of nuclei from Hepatoma 31 at pH values from 3.0 to 4.0. Instead it was necessary to employ concentrations of citric acid near to the concentrations employed by Stoneburg (4) and Marshak (5) in isolating the nuclei.

100 gm. of frozen tumor free of necrotic material were added rapidly to a Waring blender containing 500 cc. of a mixture of crushed ice, distilled water, and 100 cc. of molar citric acid. (The concentration of citric acid in this mixture is about 4 per cent, and the final pH after addition of the tumor is about 2.4.) The blender was allowed to run for 15 minutes, and then the nuclei were isolated as usual, care being taken to add a drop or so of citric acid when needed after the first washing to prevent agglutination. Agglutination is much more troublesome with nuclei prepared with strong citric acid than with nuclei prepared at pH 3.8 to 4.0.

A photograph of nuclei isolated from cells of Hepatoma 31 is shown in Fig. 2.

*Preparation of Cells of Normal Rat Liver with 4 Per Cent Citric Acid*—The same method was used which has just been described for the isolation of nuclei from Hepatoma 31. The pH is 2.4. A photograph of these nuclei is shown in Fig. 3. Some of the nuclei have been fragmented. As a rule, this does not happen.

*Preparation of Nuclei of Chicken Erythrocytes*—The method of Dounce and Lan (6) was employed, saponin being used to lake the cells in 0.9 per



FIG 2 Nuclei isolated at pH 2.4 from Hepatoma 31  $\times 1040$

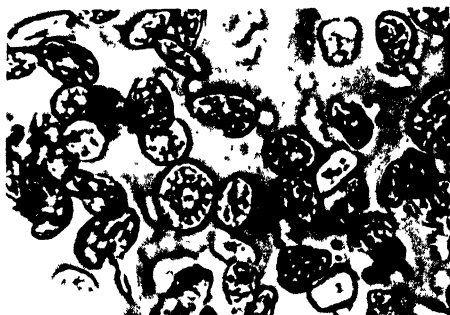


FIG 3. Nuclei isolated from rat liver (Osborne-Mendel) at pH 2.4.  $\times 1040$

cent sodium chloride solution. A photograph of these nuclei will appear elsewhere.

*Analysis of Nuclei for Desoxyribonucleic Acid*—Desoxyribonucleic acid was determined by the method already described in the preceding paper. Samples of isolated nuclei were thoroughly suspended in distilled water and two 0.5 cc. aliquots were taken for the analysis for nucleic acid. Usually two 0.5 cc. aliquots also were dried in an oven at  $100^{\circ}$  for dry weight determination, although occasionally only one 0.5 cc. sample was dried. The values for the nucleic acid analyses are shown in Table I, and are expressed as nucleic acid, not as the sodium salt.

## DISCUSSION

The results of the analyses of various samples of isolated nuclei for desoxyribonucleic acid shown in Table I are all averages of at least two determinations on a given sample of nuclei, and different preparations of nuclei were made for each result recorded. In making a given preparation of isolated nuclei of liver cells, we used from ten to fifteen rat livers, and for nuclei from the tumor cells, parts of at least three tumors were used for every preparation of nuclei.

It should be clear from Table I that the total desoxyribonucleic acid content of the nuclei of both tumors studied is not higher than the nucleic

TABLE I  
*Desoxyribonucleic Acid in Cell Nuclei, As Per Cent of Dry Weight*  
Lipid was present unless otherwise stated.

Normal rat liver, pH 3.8-4.0	Normal rat liver, 1.28 per cent citric acid, pH 3.0	Normal rat liver, 4 per cent citric acid, pH 2.4	Walker tumor, 1.5 per cent citric acid, pH 3.0	Hepatoma 31, 4 per cent citric acid, pH 2.4	Chicken red blood cell, pH 6.8-7.0	Fish sperma- tozoa
22.6*	25.3*	38.3*	21.0†	21.8*	38.1†	73.0§
20.2*			21.6†	25.3*	37.8	
23.9*		40.3†		15.2*		
				21.9*	42.0	
22.4†				22.4*		
19.7†						

\* Rats of the Osborne-Mendel strain were used.

† Rats of the Wistar strain were used.

‡ The value of 45 per cent reported previously (6) was based on our sodium thymonucleate standard obtained by the Hammarsten method without correction for impurity in the standard, and therefore is too high.

§ Value reported by Steudel (7), based on phosphorus analysis, lipid-free dry weight basis.

|| Value reported by Ackerman (8), based on phosphorus analysis, lipid-free dry weight basis.

acid content of cells of normal rat liver. In the case of Hepatoma 31, it is probable that the nucleic acid content actually is much lower than the values recorded in Table I, and therefore is also much lower than the values for nuclei isolated from normal liver cells, since the use of 4 per cent citric acid undoubtedly results in the extraction of much of the histone present in the nuclei.

This is demonstrated indirectly by the analysis of nuclei of normal liver cells, prepared at pH 2.4 by the use of 4 per cent citric acid, and at pH 3.0 by the use of 1.28 per cent citric acid. The per cent of desoxyribonucleic acid in nuclei prepared at pH 2.4 is about 1.8 times the per cent of desoxy-

ribonucleic acid in nuclei prepared at pH 3.8 to 4.0, while the per cent of desoxyribonucleic acid in nuclei prepared at pH 3.0 is about 1.17 times the per cent of desoxyribonucleic acid in nuclei prepared at pH 3.8 to 4.0. These factors are calculated from average values for the per cent of desoxyribonucleic acid in the nuclei of cells of normal rat liver prepared at pH 2.4, pH 3.0, and pH 3.8 to 4.0. If we divide the average value for the per cent of desoxyribonucleic acid in nuclei of Hepatoma 31, prepared at pH 2.4, by the factor 1.8, we get a "corrected" value of about 11.8 per cent. If we divide the average value for the per cent of desoxyribonucleic acid in Walker Carcinosarcoma 256 by the factor 1.17, we get a "corrected" value of about 18.2 per cent. It is not possible to state how close these "corrected" values for the desoxyribonucleic acid content of the tumor nuclei are to the true values, since we do not know how the histone content of the tumor cell nuclei compares with the histone content of cell nuclei of normal liver.

All of the work in this paper probably should be considered as dealing with nuclei in the resting stage, since even in the tumors the percentage of cells undergoing mitosis does not appear to be very high.

While we do not wish to contradict the observations of Koller, who used different tumor cells, a different technique, and who referred mainly to chromosomes rather than to whole nuclei, we should like to point out that his conclusions may be too sweeping, particularly in regard to the consideration of an enhanced nucleic acid content of tumor cells as a cause of malignancy.

#### SUMMARY

1. Nuclei isolated from Walker Carcinosarcoma 256 at pH 3.0 with 1.5 per cent citric acid, and nuclei isolated from Hepatoma 31 with 4 per cent citric acid have been analyzed for desoxyribonucleic acid, and the results have been compared with similar analyses of cell nuclei from normal rat liver, chicken erythrocytes, and fish spermatozoa.

2. Values obtained for the desoxyribonucleic acid content of nuclei of the Walker tumor cells are probably not greatly in error, but values obtained for nuclei isolated from Hepatoma 31 undoubtedly are too high because of extraction of histone from the nuclei by the strong acid.

3. The nuclei of Walker tumors in the resting stage have nearly the same concentration of desoxyribonucleic acid as the nuclei of cells of normal rat liver in the resting stage, while nuclei of Hepatoma 31 appear to have a much lower desoxyribonucleic acid content than this.

4. Nuclei of bird erythrocytes and fish spermatozoa have a much higher concentration of desoxyribonucleic acid than do nuclei of the two tumors studied, or the cell nuclei of normal rat liver.

We wish to express our gratitude to The International Cancer Research Foundation of Philadelphia, Pennsylvania, whose financial support has made this work possible.

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# INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES\*

## XV. SOME PHYSICAL PROPERTIES OF $l(+)$ -ALANINE AND $d(-)$ -ALANINE†

BY MAX S. DUNN, M. PALMER STODDARD,‡ LOUIS B. RUBIN,  
AND RUSSELL C. BOVIE

(From the Chemical Laboratory, University of California, Los Angeles)

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An investigation of some physical properties of  $l(+)$ -alanine and  $d(-)$ -alanine is reported in this paper. In general, the methods employed are the same as those described in earlier publications on  $l(-)$ -leucine (2) and  $l(-)$ -histidine (3). In these earlier studies, it was not difficult to acquire adequate quantities of the crude natural amino acid. Since this was not the case in the present work, it was first necessary to select a method for the preparation of the amino acid.

Although the natural antipode,  $l(+)$ -alanine, has been isolated from hydrolysates of proteins, such as gelatin and fibroin, by the ester method (Fischer (4-6)) and by means of its slightly soluble salts, the dioxypyridate (Bergmann and Niemann (7)) and the azobenzene-*p*-sulfonate (Stein *et al.* (8)), a resolution procedure was preferred, since amino acid impurities, invariably present in and difficult to remove from an amino acid isolated from a protein, would be avoided. A further advantage was that both optical antipodes, resulting from a resolution, would be available for study. By this means the reliability of the physical constants measured would be increased, since it was expected that the antipodes would have identical properties except the sign of optical rotation.

The methods employed in the preparation of  $l(+)$ -alanine include (a) the enzymatic degradation of *dl*-alanine with taka-diaxase (Hoppert (9)) and *d*-amino acid oxidase (Behrens (10)), (b) amination of the corresponding optically active  $\alpha$ -bromopropionic acid (Levene and Kuna (11)), and (c) reduction of  $l$ - $\alpha$ -amino- $\beta$ -chloropropionic acid (Fischer and Raske (12)).  $d(-)$ -Alanine has been prepared by (a) the enzymatic degradation of *dl*-alanine with *Aspergillus niger* (Fischer (13)) and yeast (Ehrlich (14)) and

\* The word "quantitative" has been omitted from the title formerly used.

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‡ Present address, Gelatin Products Company, Detroit, Michigan.



(b) amination of the corresponding  $\alpha$ -bromopropionic acid (Warburg (15), Fischer (16)). Behrens' procedure has been confirmed in the authors' laboratory and it is considered to be satisfactory. The other methods were not tested. The intermediates employed in Fischer's laboratory were obtained by the action of phosphorus pentachloride on *l*(-)-serine methyl ester and nitrosyl bromide on *l*(+)-alanine. The literature on the resolution of *dl*- $\alpha$ -bromopropionic acid has been reviewed recently by Chadwick and Pacsu (17) who concluded that, "there is no method known by which pure *d*- or *l*- $\alpha$ -bromopropionic acid may be obtained in quantities great enough for use in synthetic experiments."

The optically active alanines have been prepared most commonly by chemical resolution of *dl*-alanine. The benzoyl (5, 13, 18-22), ethyl ester (23, 24), *p*-toluenesulfonyl (25),  $\alpha$ - and  $\beta$ -naphthalenesulfonyl (26), benzenesulfonyl (27), camphorsulfonyl (23), bromocamphorsulfonyl (23), menthoxyacetyl (20, 28), hydroxymethylenecamphor (24), and *o*-, *m*-, and *p*-nitrobenzoyl (29, 30) derivatives of the brucine (5, 13, 18-22, 25, 26, 29, 30), strychnine (5, 13, 18-22, 25, 26, 29, 30), cinchonine (30), cinchonidine (29, 30), quinine (30), and ephedrine (27) salts of *dl*-alanine have been used for this purpose. Some of the alkaloidal derivatives were found to be unsatisfactory because of unfavorable solubility relations of the enantiomorphic complexes and the formation of partial racemates or mixed crystals of the diastereoisomeric salts. It was observed by Colles and Gibson (30) that the benzoyl, *p*-toluyl, cinnamoyl, and *m*- and *p*-nitrobenzoyl derivatives of alanine are readily hydrolyzed when they are refluxed for 3 hours with constant boiling hydrochloric acid, while the benzenesulfonyl and analogous sulfonyl derivatives are incompletely hydrolyzed even under more drastic conditions. It appears, however, that resistance to hydrolysis is not of critical importance, since, according to Pacsu and Mullen (20), *l*(+)-alanine is not racemized appreciably during 24 hours refluxing with 20 per cent hydrochloric acid.

The method described by Pope and Gibson (18) in 1912 for the resolution of *dl*-alanine was finally adopted, since it appeared to be as satisfactory as any other. It was first employed in the authors' laboratory in 1935 in the preparation of 2.5 gm. of purified *l*(+)-alanine and 6.5 gm. of purified *d*(-)-alanine. In this procedure, the strychnine salt of benzoyl-*l*(+)-alanine and the brucine salt of benzoyl-*d*(-)-alanine are isolated in the order given. The precipitation of these complexes in the reverse order, according to the method devised originally by Fischer (13), is not as satisfactory as the procedure of Pope and Gibson, since the strychnine salt of *l*(+)-benzoylalanine is much less soluble in water than the brucine salt of *d*(-)-alanine. Although Pope and Gibson did not extend the resolution beyond the benzoylalanine stage, Pacsu and Mullen (20), as well as Levy and Palmer

(21), have reported recently the preparation of both alanine antipodes essentially by the method of Pope and Gibson.

*Preparation of Purified l(+)-Alanine and d(-)-Alanine*

**Benzoyl-dl-alanine**—A mixture consisting of 267 gm. (3 moles) of technical dl-alanine, 500 cc. of distilled water, and 175 cc. of saturated sodium hydroxide solution was cooled to about 0°. 360 cc. (3.12 moles) of benzoyl chloride were added rapidly while the mixture was stirred. Ice and alkali solution (125 cc.) were added as needed to maintain the temperature low and the reaction alkaline to phenolphthalein. 200 cc. of concentrated hydrochloric acid were added, and the mixture was allowed to stand overnight in the refrigerator. The suspension of benzoic acid and benzoyl-dl-alanine was filtered and the precipitate washed with 1.5-liters of cold distilled water and 600 cc. of isopropyl ether. The yield of product (m.p. 160–161°, uncorrected), dried in air, was 550 to 557 gm. (95 to 97 per cent of theory). According to Pope and Gibson (18), the melting point is 160° (uncorrected), while Fischer (13) found 162–163° (uncorrected) and 165–166° (corrected).

Because he was not able to get satisfactory yields of benzoyl-dl-alanine by the original method of Baum (31), Fischer (13) substituted sodium bicarbonate for sodium hydroxide in the benzoylation. By this means he prepared 6.0 gm. of crude and 4.5 gm. of once recrystallized product in 97 and 73 per cent, respectively, of the theoretical amount. Carter and Stevens (32) found that the yield of the purified benzoyl derivative of l-p-methoxyphenylalanine was higher when sodium hydroxide was employed in place of sodium bicarbonate and benzoyl chloride was used in the ratio of 2 moles to 1 of the amino acid instead of the 3:1 ratio recommended by Fischer. Adopting this procedure, Levy and Palmer (21) obtained 38 gm. (79 per cent of the theoretical amount) of benzoyl-dl-alanine which was stated to have the theoretical titration equivalent and the accepted melting point. By the present authors' relatively simple procedure only a slight excess of benzoyl chloride is required and large amounts of nearly pure benzoylated product are readily prepared in high yield.

**Strychnine Benzoyl-l(+)-alanine**—The procedure of Pope and Gibson (18) was followed in the resolution of benzoyl-dl-alanine. Strychnine benzoyl-l(+)-alanine is allowed to crystallize from an aqueous solution containing 2 equivalents of benzoyl-dl-alanine and 1 equivalent each of potassium hydroxide and strychnine. It was found, in agreement with Levy and Palmer (21), that this method is as effective and is more economical of alkaloid than that of Fischer (13) as modified by Pacsu and Mullen (20). The last authors crystallized strychnine benzoyl-l(+)-alanine from an aqueous solution containing equivalent quantities of benzoyl-dl-alanine and strychnine.

From seven lots (109 to 177 gm.) of benzoyl-*dl*-alanine (total, 1064 gm.) there were obtained 1239.7 gm. (79.9 per cent of theory) of recrystallized *l*-strychnine benzoyl-*l*(+)-alanine which, according to Pope and Gibson (18), is the dihydrate. The specific rotation (Sample A, Table I) in water was  $-10.45^\circ$  at  $24^\circ$ . That of Pope and Gibson's product (Sample B, Table I) was  $-10.66^\circ$  at  $20^\circ$ . Pacsu and Mullen (20) prepared 310.7 gm. (79.5 per cent of theory) of this salt. These authors calculated the yield to be 84.6 per cent of theory based, apparently, on the assumption that the salt is anhydrous.

*Brucine Benzoyl-d(-)-alanine*—The mother liquor from which the strychnine benzoyl-*l*(+)-alanine had been removed was evaporated to about one-half of its volume. The residual solution was made strongly alkaline with 3 *N* sodium hydroxide, the mixture was cooled overnight in the refrigerator, the suspension of strychnine was filtered, the precipitate was washed, and the filtrate was acidified to Congo red with concentrated hydrochloric acid. The precipitate of crude benzoyl-*d*(-)-alanine, which formed immediately, was removed by filtration. Two additional crops of crystals were obtained from the filtrate. The yield of crude benzoyl-*d*(-)-alanine was 443.2 gm. (83 per cent of theory).

The crude product (2.06 moles, if 90 per cent pure) was dissolved in 4.5 liters of boiling water and there were added 820 gm. (2.08 moles) of anhydrous *l*-brucine and 12.9 gm. (0.23 mole) of potassium hydroxide dissolved in 100 cc. of water. The resulting solution was cooled, seeded, and let stand for 36 hours in the refrigerator. The suspension of fine crystals was filtered. The crystals were washed twice with ice-cold water and dried at  $50^\circ$ . The yield<sup>1</sup> (brucine benzoyl-*d*(-)-alanine· $4\frac{1}{2}$ H<sub>2</sub>O, according to Pope and Gibson (18)) was 1059 gm. (69.1 per cent of theory based on 443.2 gm. of crude benzoyl-*d*(-)-alanine and 71 per cent based on 1064 gm. of benzoyl-*dl*-alanine). The yield of twice recrystallized product was 890 gm. (84 per cent recovery). The crude product melted at  $84-86^\circ$  (uncorrected) and the final product at  $86-88^\circ$  (uncorrected). Pope and Gibson (18) found that a product which had been recrystallized four times melted at  $89-91^\circ$ . The specific rotation in water of the authors' product (Sample C, Table I) was  $-26.53^\circ$  at  $23.0^\circ$  and Pope and Gibson's product (Sample D, Table I) was  $-33.6^\circ$  at  $20^\circ$ .

*Benzoyl-l(+)-alanine*—A hot aqueous solution containing 1120.9 gm. (1.99 moles) of once recrystallized strychnine benzoyl-*l*(+)-alanine was made strongly alkaline with 3 *N* sodium hydroxide. The mixture was cooled

<sup>1</sup> Pacsu and Mullen (20) prepared 359 gm. of the brucine salt. The yield, 88.2 per cent, calculated by these authors seems to be in error, since it appears to be 74 per cent based on 140 gm. of benzoyl-*d*(-)-alanine and 77.7 per cent based on 268 gm. of benzoyl-*dl*-alanine.

and the suspension of strychnine was filtered immediately. The filtrate was acidified strongly with concentrated hydrochloric acid, seeded, and let stand overnight in the refrigerator. The resulting crystals were removed by filtration, washed, and dried at 50°. The yield of crude benzoyl-*l*(+)-alanine (m.p. 136–139°, uncorrected) was 379.7 gm. (99 per cent of theory).

The crude benzoyl-*l*(+)-alanine was suspended in 2.5 liters of water heated to 40°, 121 cc. of concentrated sodium hydroxide and 9 gm. of norit were added, and the mixture was stirred for 10 minutes. The suspension was filtered, and the precipitate was washed. Concentrated hydrochloric acid (215 cc.) was added to the filtrate and the suspension which formed after the solution had stood overnight was filtered. The crystals were washed with cold water, sucked as dry as possible, and dried at 50°. There were obtained from 326.3 gm. of crude benzoyl-*l*(+)-alanine 321.5 gm. (98.5 per cent recovery) of recrystallized product which melted at 136–138° (uncorrected). Its titration equivalent was 189.2 (theory, 193.1) and the moisture content was 1.5 per cent. The pure substance melts at 147–148° (uncorrected) or 150–151° (corrected) according to Fischer (13), Pope and Gibson (18), and Pacsu and Mullen (20). The specific rotation (Sample E, Table I) was +33.4° in 1 *N* sodium hydroxide at 22.5°. That of Pope and Gibson's product (Sample F, Table I) was +34.6° in 0.077 *N* potassium hydroxide at 20°, and of Pacsu and Mullen's product (Sample G, Table I) was +37.12° in 0.34 *N* potassium hydroxide at 20°. Although Pope and Gibson, Pacsu and Mullen, and Levy and Palmer (21) have shown that the specific rotation of benzoyl-*l*(+)-alanine in an aqueous solution containing an equivalent of alkali varies with the concentration of the solute, it is difficult to determine from the available data the correct rotation under any particular set of conditions.

*Benzoyl-d*(-)-alanine<sup>2</sup>—1337 gm. (2.00 moles) of *l*-brucine benzoyl-*d*(-)-alanine·4½H<sub>2</sub>O were dissolved in 5 liters of water and 480 cc. of saturated sodium hydroxide solution were added. The suspension of brucine, which formed rapidly, was stirred for 10 minutes, cooled to room temperature, and filtered. The brucine was washed with 0.5 *N* sodium hydroxide solution and the combined filtrate and washings were acidified to Congo red with 340 cc. of concentrated c.p. hydrochloric acid. The suspension was filtered after it had stood overnight in the refrigerator. The crystals were washed with cold water and dried for 2 days at 50°.

The yield of crude benzoyl-*d*(-)-alanine was 364.9 gm. (94.5 per-cent of theory). The specific rotation (Sample H, Table I) was -32.5° in 1.05

<sup>2</sup> Pacsu and Mullen (20) prepared 77.5 gm. of purified benzoyl-*d*(-)-alanine. The yield was 91 per cent of theory based on brucine benzoyl-*d*(-)-alanine·4½H<sub>2</sub>O. The yield, 81 per cent, given by these authors apparently was calculated on the assumption that brucine benzoyl-*d*(-)-alanine is anhydrous.

N sodium hydroxide at 25°. The values,  $-37.3^{\circ}$  and  $-36.9^{\circ}$  at  $20^{\circ}$ , were found by Fischer (13) and Pacsu and Mullen (20), respectively, under approximately the same conditions.

*l*(+)-Alanine—292.5 gm. (1.51 moles) of benzoyl-*l*(+)-alanine suspended in 1460 cc. of 6 N hydrochloric acid were heated for 4.5 hours on a boiling water bath. The mixture was cooled overnight in the refrigerator, 1 liter of distilled water was added, and the suspension of crystals was filtered. After the benzoic acid was extracted from the precipitate, about 40 gm. of unchanged benzoyl-*l*(+)-alanine were recovered.

The acid filtrate was distilled to dryness *in vacuo* and, after the addition of 300 cc. of water, this process was repeated twice. The residual *l*(+)-alanine hydrochloride was dissolved in 400 cc. of distilled water, 89 cc. of concentrated ammonium hydroxide and 293 cc. of 95 per cent ethanol were added, and the suspension of crystals which formed after the mixture had stood overnight in the refrigerator was filtered. The crystals were washed with 95 per cent ethanol until the washings were free from chloride. The yield of *l*(+)-alanine, dried at  $50^{\circ}$ , was first crop, 65.5 gm., second crop, 9.3 gm., and total 74.8 gm. (64 per cent of theory). The specific rotations in 5.97 N hydrochloric acid at  $25^{\circ}$  were first crop (Sample I, Table I),  $+13.84^{\circ}$ , and second crop (Sample J, Table I),  $+13.73^{\circ}$ .

72 gm. of the foregoing *l*(+)-alanine were dissolved in 214 cc. of boiling distilled water and 520 cc. of 95 per cent ethanol were added. The yield of dried product was 66.9 gm. (93 per cent recovery). It contained less than 0.004 per cent chloride, phosphate, iron, or heavy metal ion, and it was 100.6 per cent pure according to Van Slyke volumetric analysis of amino nitrogen. Its specific rotation (Sample K, Table I) in 6.08 N hydrochloric acid at  $25.0^{\circ}$  was  $+13.83^{\circ}$ .

53.2 gm. of this product were dissolved in 162 cc. of boiling distilled water and 380 cc. of 95 per cent ethanol were added. The yield of dried product was 49.8 gm. (93.6 per cent recovery). Its specific rotation (Sample L, Table I) in 5.97 N hydrochloric acid at  $25.1^{\circ}$  was  $+13.79^{\circ}$ .

47.8 gm. of this product were dissolved in 150 cc. of boiling distilled water and 172 cc. of redistilled absolute methanol were added. The yield of dried product containing less than 0.004 per cent chloride, ammonium, iron, phosphate, or heavy metal ion was 40.3 gm. (84.3 per cent recovery). The product was 100.3 (100.2, 100.3, 100.3) per cent pure according to semi-micro-Kjeldahl analysis. Its specific rotation (Sample M, Table I) in 5.97 N hydrochloric acid at  $25.0^{\circ}$  was  $+13.82^{\circ}$ .

3.0 gm. of this product were dissolved in 9.0 cc. of boiling distilled water. This solution was cooled at  $5^{\circ}$  for 4 hours, the suspension of crystals was filtered, and the crystals were washed with 33 per cent, 50 per cent, and absolute methanol. The yield of dried product was 1.63 gm. (54.3 per

cent recovery). The specific rotation (Sample N, Table I) in 5.97 N hydrochloric acid at 25.0° was +13.70°. About 14 cc. of absolute methanol were added to the alcoholic filtrate and the yield of dried product which

TABLE I  
*Specific Rotations\* of Substances Referred to in Text of This Paper†*

Sample	Substance	Solvent	Weight of sample	Volume of solution	Temperature	$\alpha$	$[\alpha]_D$
			gm.	cc.	°C.	degrees	degrees
A	<i>l</i> -Strychnine benzoyl- <i>l</i> (+)-alanine dihydrate	Water	0.2154	50.04	24	-0.180	-10.45
B	" "	"	0.1088	29.94	20	-0.155	-10.66‡
C	<i>l</i> -Brucine benzoyl- <i>d</i> (-)-alanine·4½ H <sub>2</sub> O	"	0.5314	50.01	23.0	-1.127	-26.53
D	" "	"	0.3117	29.94	20	-1.40	-33.6‡
E	Benzoyl- <i>l</i> (+)-alanine	1 N NaOH	0.2301	50.01	22.5	+0.614	+33.4
F	"	0.077 N KOH	0.5238	29.94	20	+2.42	+34.6‡
G	"	0.34 " "	0.6681	10	20	+2.48	+37.12§
H	Benzoyl- <i>d</i> (-)-alanine	1.05 " NaOH	0.5008	50.0	25	-1.366	-32.5
I	<i>l</i> (+)-Alanine	5.97 " HCl	0.5309	26.293	25	+1.118	+13.84
J	"	5.97 " "	0.5352	21.131	25	+1.125	+13.73
K	"	6.08 " "	0.5447	26.044	25.0	+1.157	+13.83
L	"	5.97 " "	0.5281	26.293	25.1	+1.108	+13.79
M	"	5.97 " "	0.5556	26.131	25.0	+1.175	+13.82
N	"	5.97 " "	0.5256	26.206	25.0	+1.099	+13.70
O	"	5.97 " "	0.5422	26.293	25.1	+1.130	+13.70
P	<i>d</i> (-)-Alanine	6.08 " "	0.5436	22.044	25.0	-1.333	-13.57
Q	"	5.97 " "	0.5303	26.044	25.1	-1.107	-13.59
R	"	5.97 " "	0.4312	26.126	25.0	-1.111	-13.66
S	"	5.97 " "	0.5255	25.949	25.0	-1.103	-13.62
T	"	5.97 " "	0.5306	26.044	25.2	-1.107	-13.58

\* A 1 dm. tube was used to measure the rotation of benzoyl-*l*(+)-alanine, Sample G. In all other cases the tube length was 4 dm.

† The data given were obtained by the present authors with the exceptions noted.

‡ Pope and Gibson (18).

§ Pacsu and Mullen (20).

crystallized at 5° was 0.80 gm. The specific rotation (Sample O, Table I) in 5.97 N hydrochloric acid at 25.1° was +13.70°.

Since all of the foregoing seven specific rotations are either within, or only slightly beyond, the probable precision of the polarimeter, it was considered that this sample of *l*(+)-alanine was analytically pure, its specific

rotation being  $[\alpha]_D^{25.0} = +13.77^\circ (\pm 0.02^\circ, \text{probable error of the mean})$  in 6.0 N hydrochloric acid.

*d*(-)-Alanine—230 gm. (1.19 moles) of recrystallized benzoyl-*d*(-)-alanine were hydrolyzed with concentrated hydrochloric acid, the suspension of benzoic acid filtered, and *d*(-)-alanine isolated from the filtrate essentially by the method employed in the preparation of *l*(+)-alanine from benzoyl-*l*(+)-alanine. The yield of crude *d*(-)-alanine was first crop, 76.2 gm., second crop, 18.7 gm., third crop, 6.4 gm., and total, 101.3 gm. (95.8 per cent of theory).

74.7 gm. of this product were recrystallized from aqueous ethanol solution. The yield of dried product containing less than 0.004 per cent chloride, phosphate, iron, or heavy metal ion was 69.6 gm. (93.2 per cent recovery). This product was 100.8 per cent pure according to Van Slyke volumetric analysis of amino nitrogen. Its specific rotation (Sample P, Table I) in 6.08 N hydrochloric acid at 25.0° was  $-13.57^\circ$ .

54.1 gm. of this product were recrystallized from aqueous ethanol solution. The yield of dried product was 50.5 gm. (93.3 per cent recovery). The specific rotation (Sample Q, Table I) in 5.97 N hydrochloric acid at 25.1° was  $-13.59^\circ$ .

50.1 gm. of this product were recrystallized from aqueous methanol solution. The yield of dried product containing less than 0.004 per cent chloride, ammonia, iron, phosphate, or heavy metal ion was 42.8 gm. (85.4 per cent recovery). This product was 100.2 (100.3, 100.4, 100.0) per cent pure according to semimicro-Kjeldahl analysis. Its specific rotation (Sample R, Table I) in 5.97 N hydrochloric acid at 25.0° was  $-13.66^\circ$ .

3.0 gm. of this product were recrystallized from aqueous methanol solution. The yield of dried product was 1.65 gm. (55.0 per cent recovery). The specific rotation (Sample S, Table I) in 5.97 N hydrochloric acid at 25.0° was  $-13.62^\circ$ . 30 cc. of absolute methanol were added to the alcoholic filtrate and the mixture let stand at 5°. The yield of dried product was 0.87 gm. The specific rotation (Sample T, Table I) in 5.97 N hydrochloric acid at 25.2° was  $-13.58^\circ$ .

Since all of the foregoing five specific rotations are either within, or only slightly beyond, the probable precision of the polarimeter, it was considered that this sample of *d*(-)-alanine was analytically pure, its specific rotation being  $[\alpha]_D^{25.0} = -13.60^\circ (\pm 0.01^\circ, \text{probable error of the mean})$  in 6.0 N hydrochloric acid.

#### *Purity of Purified l(+)-Alanine and d(-)-Alanine*

The average specific rotations,  $+13.77^\circ$  and  $-13.60^\circ$ , found under the specified conditions ( $c = 1$ ,  $t = 25$ , and  $\text{HCl} = 5.97 \text{ N}$ ) for the purified *l*(+)-alanine and *d*(-)-alanine, differ by 1.2 per cent. Since the moisture,

ash, or inorganic ion impurity (determined by the methods described by Stoddard and Dunn (2)) was less than 0.01 per cent, it was difficult to account for a difference in specific rotation of this magnitude on the basis of any known probable error. For this reason, the additional experiments described below were performed in order to determine as accurately as possible the purity of these samples of the alanine antipodes.

The specific rotations of the *l*(+)-alanine and *d*(-)-alanine were determined under the conditions shown in Table II. It may be noted that the average deviation of the recorded values for the antipodes at the experimental temperatures, 0–45°, and the solvents, water and hydrochloric acid (0.2 to 7.25 *N*), is 0.75 per cent. It is evident, also, that the deviations under optimum conditions are less than 0.5 per cent. It appears probable, therefore, that the differences in specific rotations observed initially fall within the limits of probable error inherent in measurements under the specified conditions.

A dependable criterion of purity of optical isomers is believed to be the concordance of solubilities with small and large excesses of solute when mixed crystal formation does not occur. The solubilities of the alanines were measured by the gravimetric method described in the authors' earlier publications. In the present work, 10 cc. glass-stoppered conical flasks were found to be more satisfactory receptacles for the weighing and the evaporation of the aliquots of the saturated solutions than Petri dishes or glass-stoppered weighing bottles. The latter are unsatisfactory because of the tendency of the solution to creep up the sides and over the top of the vertical walls during the evaporation.

The experimental conditions and results are shown in Table III. It may be observed that the solubilities of *l*(+)-alanine and *d*(-)-alanine at 25.0° in water are 16.505 (0.0045, probable error of the mean) and 16.490 (0.012, probable error of the mean), respectively. These mean values differ by only 0.03 per cent and are in close agreement with the value, 16.62, reported by Pellini and Coppola (33) (obtained by interpolation from a curve constructed from a plot of these authors' data at 0°, 17°, 30°, and 45°) and 16.65, found by Dalton and Schmidt (34).

The purity of the authors' specimens was tested finally as follows: Distilled water was boiled, cooled, and stored in a scrupulously cleaned container fitted with a soda lime tube and a siphon. Mixtures of the alanine antipodes containing approximately 99, 95, 5, or 1 per cent of each of the isomers were prepared by weighing accurately and mixing appropriate quantities of the alanines. A quantity of each mixture, equivalent approximately to 110 to 130 per cent of the amount of pure antipode calculated to be soluble in the volume of water taken, was suspended in the specially prepared distilled water. The solubilities of these mixtures were



**TABLE II**  
*Specific Rotations of l(+)-Alanine and d(-)-Alanine\* (Authors' Data)*

Alanine isomer	Solution		Sample	Solute		Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^t$ (specific rotation) $\dagger$
	Volume	$d$ (density) $\dagger$		$c$ (per 100 cc solution)	$\phi$ (per 100 gm. solution)			
7.25 N hydrochloric acid ( $c = 2$ )								
	cc.		gm.	gm.	gm.	°C.	degrees	degrees
<i>l</i> (+)	26.011	1.1286	0.5299	2.037	1.806	0.50	+1.249	+15.33
<i>d</i> (-)	25.925	<b>1.1286</b>	0.5302	2.045	1.812	0.50	-1.235	-15.10
<i>l</i> (+)	26.178	1.1214	0.5299	2.024	1.806	15.0	+1.159	+14.31
<i>d</i> (-)	26.092	<b>1.1214</b>	0.5302	2.032	1.812	15.0	-1.143	-14.06
<i>l</i> (+)	26.293	1.1165	0.5299	2.015	1.806	25.0	+1.094	+13.57
<i>d</i> (-)	26.206	<b>1.1165</b>	0.5302	2.023	1.812	25.0	-1.079	-13.33
<i>l</i> (+)	26.409	1.1116	0.5299	2.006	1.806	35.0	+1.033	+12.87
<i>d</i> (-)	26.322	<b>1.1116</b>	0.5302	2.014	1.812	35.0	-1.021	-12.67
<i>l</i> (+)	26.528	1.1066	0.5299	1.998	1.806	45.0	+0.977	+12.23
<i>d</i> (-)	26.440	<b>1.1066</b>	0.5302	2.005	1.812	45.0	-0.966	-12.04
5.97 N hydrochloric acid ( $c = 10$ )								
<i>l</i> (+)	25.944	1.1192	2.6275	10.127	9.049	0.50	+6.616	+16.33 (+16.32)
<i>d</i> (-)	25.953	1.1193	2.6304	10.135	9.058	0.50	-6.581	-16.23 (-16.22)
<i>l</i> (+)	26.092	1.1129	2.6275	10.070	9.049	15.0	+6.116	+15.18 (+15.17)
<i>d</i> (-)	26.102	1.1129	2.6304	10.078	9.058	15.0	-6.089	-15.10 (-15.09)
<i>l</i> (+)	26.195	1.1085	2.6275	10.030	9.049	25.0	+5.805	+14.47
<i>d</i> (-)	26.206	1.1085	2.6304	10.037	9.058	25.0	-5.786	-14.41
<i>l</i> (+)	26.305	1.1039	2.6275	9.989	9.049	35.0	+5.504	+13.78
<i>d</i> (-)	26.315	1.1039	2.6304	9.996	9.058	35.0	-5.484	-13.72
<i>l</i> (+)	26.415	1.0993	2.6275	9.947	9.049	45.0	+5.221	+13.12
<i>d</i> (-)	26.430	1.0991	2.6304	9.952	9.058	45.0	-5.204	-13.07
5.97 N hydrochloric acid ( $c = 6$ )								
<i>l</i> (+)	26.040	1.1136	1.5527	5.9627	5.3556	0.50	+3.805	+15.95
<i>d</i> (-)	25.792	1.1139	1.5759	6.1100	5.4881	0.50	-3.888	-15.91 (-15.90)
<i>l</i> (+)	26.193	1.1071	1.5527	5.9279	5.3556	15.0	+3.509	+14.81 (+14.82)
<i>d</i> (-)	25.945	1.1073	1.5759	6.0739	5.4881	15.0	-3.589	-14.77 (-14.76)
<i>l</i> (+)	26.293	1.1029	1.5527	5.9054	5.3556	25.0	+3.320	+14.06 (+14.07)
<i>d</i> (-)	26.044	1.1031	1.5759	6.0508	5.4881	25.0	-3.395	-14.03
<i>l</i> (+)	26.402	1.0983	1.5527	5.8810	5.3556	35.0	+3.168	+13.47 (+13.48)
<i>d</i> (-)	26.152	1.0986	1.5759	6.0260	5.4881	35.0	-3.217	-13.35
<i>l</i> (+)	26.521	1.0934	1.5527	5.8546	5.3556	45.0	+2.990	+12.77 (+12.78)
<i>d</i> (-)	26.266	1.0938	1.5759	5.9998	5.4881	45.0	-3.051	-12.71
5.97 N hydrochloric acid ( $c = 3.5$ )								
<i>l</i> (+)	25.872	1.1107	0.9211	3.5602	3.2069	0.50	+2.237	+15.71 (+15.70)
"	26.020	1.1044	0.9211	3.5400	3.2069	15.0	+2.062	+14.56
"	26.126	1.0999	0.9211	3.5256	3.2069	25.0	+1.954	+13.86
"	26.233	1.0954	0.9211	3.5112	3.2069	35.0	+1.854	+13.20
"	26.372	1.0897	0.9211	3.4927	3.2069	45.0	+1.761	+12.60

TABLE II—Continued

Alanine isomer	Solution		Sample	Solute		Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^{25}$ (specific rotation)†
	Volume	$d$ (density)†		$c$ (per 100 cc. solution)	$\phi$ (per 100 gm. solution)			
4.83 N hydrochloric acid ( $c = 2$ )								
	cc.		gm.	gm.	gm.	°C.	degrees	degrees
<i>l</i> (+)	26.069	1.0896	0.5286	2.028	1.861	0.50	+1.270	+15.66
<i>d</i> (-)	25.820	1.0897	0.5315	2.058	1.889	0.50	-1.297	-15.75 (-15.74)
<i>l</i> (+)	26.198	1.0842	0.5286	2.018	1.861	15.0	+1.179	+14.61
<i>d</i> (-)	25.951	1.0842	0.5315	2.048	1.889	15.0	-1.197	-14.61
<i>l</i> (+)	26.293	1.0803	0.5286	2.010	1.861	25.0	+1.121	+13.94
<i>d</i> (-)	26.044	1.0803	0.5315	2.041	1.889	25.0	-1.134	-13.89
<i>l</i> (+)	26.398	1.0760	0.5286	2.002	1.861	35.0	+1.066	+13.31
<i>d</i> (-)	26.148	1.0760	0.5315	2.033	1.889	35.0	-1.075	-13.22
<i>l</i> (+)	26.497	1.0720	0.5286	1.996	1.861	45.0	+1.012	+12.68
<i>d</i> (-)	26.256	1.0716	0.5315	2.024	1.889	45.0	-1.027	-12.68
0.884 N hydrochloric acid ( $c = 8$ )								
<i>l</i> (+)	26.164	1.0357	2.0700	7.912	7.643	0.50	+4.745	+14.99 (+15.00)
<i>d</i> (-)	26.166	1.0358	2.0712	7.916	7.641	0.50	-4.753	-15.01 (-15.02)
<i>l</i> (+)	26.227	1.0332	2.0700	7.893	7.643	15.0	+4.427	+14.02 (+14.03)
<i>d</i> (-)	26.227	1.0334	2.0712	7.897	7.641	15.0	-4.423	-14.00 (-14.01)
<i>l</i> (+)	26.293	1.0306	2.0700	7.873	7.643	25.0	+4.234	+13.45 (+13.46)
<i>d</i> (-)	26.293	1.0308	2.0712	7.877	7.641	25.0	-4.220	-13.39 (-13.40)
<i>l</i> (+)	26.383	1.0271	2.0700	7.846	7.643	35.0	+4.037	+12.86 (+12.87)
<i>d</i> (-)	26.383	1.0273	2.0712	7.851	7.641	35.0	-4.029	-12.83 (-12.84)
<i>l</i> (+)	26.489	1.0230	2.0700	7.815	7.643	45.0	+3.848	+12.31 (+12.33)
<i>d</i> (-)	26.486	1.0233	2.0712	7.820	7.641	45.0	-3.841	-12.28 (-12.30)
0.502 N hydrochloric acid ( $c = 4.5$ )								
<i>l</i> (+)	26.193	1.0205	1.1751	4.486	4.397	0.50	+2.639	+14.71
<i>d</i> (-)	26.190	1.0205	1.1770	4.494	4.401	0.50	-2.639	-14.68
<i>l</i> (+)	26.239	1.0187	1.1751	4.478	4.397	15.0	+2.455	+13.70
<i>d</i> (-)	26.236	1.0187	1.1770	4.486	4.401	15.0	-2.575	-14.35§
<i>l</i> (+)	26.293	1.0166	1.1751	4.469	4.397	25.0	+2.342	+13.10
<i>d</i> (-)	26.293	1.0165	1.1770	4.476	4.401	25.0	-2.336	-13.05
<i>l</i> (+)	26.384	1.0131	1.1751	4.454	4.397	35.0	+2.245	+12.60
<i>d</i> (-)	26.376	1.0133	1.1770	4.462	4.401	35.0	-2.237	-12.53
<i>l</i> (+)	26.488	1.0091	1.1751	4.436	4.397	45.0	+2.132	+12.01 (+12.02)
<i>d</i> (-)	26.475	1.0095	1.1770	4.446	4.401	45.0	-2.140	-12.03
0.228 N hydrochloric acid ( $c = 2$ )								
<i>l</i> (+)	26.041	1.0095	0.5303	2.034	2.017	0.50	+1.152	+14.14
<i>d</i> (-)	26.041	1.0094	0.5309	2.039	2.018	0.50	-1.153	-14.13
<i>l</i> (+)	26.069	1.0084	0.5303	2.034	2.017	15.0	+1.083	+13.31
<i>d</i> (-)	26.074	1.0081	0.5309	2.036	2.018	15.0	-1.133	-13.91§
<i>l</i> (+)	26.126	1.0062	0.5303	2.030	2.017	25.0	+1.033	+12.72

TABLE II—*Concluded*

Alanine isomer	Solution		Sample	Solute		Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^t$ (specific rotation)†
	Volume	<i>d</i> (density)†		<i>c</i> (per 100 cc solution)	$\phi$ (per 100 gm solution)			
0.223 N hydrochloric acid ( <i>c</i> = 2)—continued								
	cc.		gm	gm.	gm	°C	degrees	degrees
<i>d</i> (-)	26.126	1.0061	0.5309	2.032	2.018	25.0	-1.018	-12.52
<i>l</i> (+)	26.209	1.0030	0.5303	2.023	2.017	35.0	+0.991	+12.24
<i>d</i> (-)	26.209	1.0029	0.5309	2.026	2.018	35.0	-0.967	-11.93
<i>l</i> (+)	26.306	0.9993	0.5303	2.016	2.017	45.0	+0.940	+11.66
<i>d</i> (-)	26.306	0.9992	0.5309	2.018	2.018	45.0	-0.923	-11.43
Water ( <i>c</i> = 10)								
<i>l</i> (+)	26.015	1.0334	2.6298	10.109	9.784	0.50	+1.360	+3.363 (+3.349)
<i>d</i> (-)	26.087	1.0349	2.7297	10.464	10.109	0.50	-1.423	-3.400 (-3.340)
<i>l</i> (+)	26.063	1.0315	2.6298	10.090	9.784	15.0	+1.125	+2.787 (+2.775)
<i>d</i> (-)	26.130	1.0332	2.7297	10.447	10.109	15.0	-1.178	-2.819 (-2.761)
<i>l</i> (+)	26.131	1.0288	2.6298	10.064	9.784	25.0	+0.974	+2.420 (+2.412)
<i>d</i> (-)	26.206	1.0302	2.7297	10.416	10.109	25.0	-1.022	-2.453 (-2.399)
<i>l</i> (+)	26.218	1.0254	2.6298	10.031	9.784	35.0	+0.807	+2.011 (+2.007)
<i>d</i> (-)	26.290	1.0269	2.7297	10.383	10.109	35.0	-0.844	-2.032 (-1.982)
<i>l</i> (+)	26.318	1.0215	2.6298	9.992	9.784	45.0	+0.627	+1.569 (+1.570)
<i>d</i> (-)	26.396	1.0228	2.7297	10.341	10.109	45.0	-0.667	-1.612 (-1.568)
Water ( <i>c</i> = 6)								
<i>l</i> (+)	26.026	1.0203	1.5897	6.108	5.984	0.50	+0.690	+2.824 (+2.810)
<i>d</i> (-)	26.095	1.0208	1.6328	6.257	6.126	0.50	-0.697	-2.785 (-2.752)
<i>l</i> (+)	26.052	1.0193	1.5897	6.102	5.984	15.0	+0.553	+2.266 (+2.253)
<i>d</i> (-)	26.139	1.0191	1.6328	6.247	6.126	15.0	-0.565	-2.261 (-2.229)
<i>l</i> (+)	26.126	1.0164	1.5897	6.085	5.984	25.0	+0.460	+1.890 (+1.879)
<i>d</i> (-)	26.195	1.0169	1.6328	6.233	6.126	25.0	-0.464	-1.861 (-1.831)
<i>l</i> (+)	26.203	1.0134	1.5897	6.067	5.984	35.0	+0.366	+1.508 (+1.499)
<i>d</i> (-)	26.278	1.0137	1.6328	6.214	6.126	35.0	-0.374	-1.505 (-1.477)
<i>l</i> (+)	26.307	1.0094	1.5897	6.043	5.984	45.0	+0.261	+1.080 (+1.074)
<i>d</i> (-)	26.379	1.0098	1.6328	6.190	6.126	45.0	-0.272	-1.099 (-1.074)

\* All rotations were measured with a 4.000 dm. water-jacketed polarimeter tube and a 0.01° Schmidt and Haensch precision polarimeter. All solutions were prepared in a thermostat at the indicated temperatures. All apparatus employed in measuring volumes and weights was accurately calibrated. The observed optical rotations were considered reliable to 0.005°. The accuracy of the recorded thermostat temperatures is  $\pm 10$  per cent at 0.50°,  $\pm 0.7$  per cent at 15.0°,  $\pm 0.4$  per cent at 25.0°,  $\pm 0.3$  per cent at 35.0°, and  $\pm 0.2$  per cent at 45.0°.

† All densities were measured at the recorded temperatures. The values set in bold-faced type were assumed to be the same as those measured for solutions of the antipode.

‡ The specific rotations shown in parentheses are the values calculated for *c* = 2.000, 3.500, 4.500, 6.000, and 10.00 by means of the factors shown in Table VI. In all other cases the experimentally determined and the calculated values are identical.

§ This figure appears to be incorrect.

determined in water at 25.0° by the gravimetric method previously employed. These data are given in Table IV.

The "theoretical" solubility data given in Table IV<sup>b</sup> were calculated on the assumption that the aqueous solution of the mixture contained at equilibrium 16.50 gm. per 100 gm. of water of the alanine antipode which was present in the mixture in excess of this previously determined average value. It was assumed, also, that all of the antipode, present in the mixture in an amount much smaller than its known solubility value in water,

TABLE III

*Solubilities of l(+)-Alanine and d(-)-Alanine in Water at 25.0° (Authors' Data)*

Excess solute	Side from which equilibrium approached	Time in thermostat	Solubility per 100 gm. water	
			l(+)-Alanine	d(-)-Alanine
<i>per cent</i>		<i>hrs.</i>	<i>gm.</i>	<i>gm.</i>
20	Cold	24	16.357*	
20	"	24	16.425*	
20	Hot	48	16.499	16.487
20	"	48	16.485	16.491
20	Cold	49	16.503	
20	"	49	16.493	
20	"	72	16.507	
20	"	72	16.554	
20	"	93	16.478	16.589
20	"	93	16.512	16.565
20	"	93	16.488	16.475
20	"	117	16.497	16.607*
20	"	117	16.516	16.577
20	"	117	16.508	16.472
40	Hot	48	16.497	16.439
40	"	48	16.530	16.395*
40	Cold	48	16.558	16.424
40	"	48	16.453	16.437
40	"	48		16.430
Arithmetic mean			16.505	16.490

\* Omitted in calculation of the mean value.

was contained in the saturated solution of the mixture. The values given under the heading "Theoretical (no *dl*)" were calculated on the assumption that the saturated aqueous solution of the mixture contained no species of alanine other than the *l*(+) or *d*(-) form. The values given under the heading "Theoretical, (*dl*)" were calculated on the assumption that the solution contained only *l*(+)- and *dl*-alanines.

It is evident from a consideration of the data given in Table IV that the saturated solutions of mixtures of *l*(+)- and *d*(-)-alanine contained at

equilibrium at 25° little, or no, *dl*-alanine. It appears, therefore, that the alanine antipodes do not undergo appreciable association under the observed experimental conditions even though, according to Dalton and

TABLE IV

*Solubility of Mixtures of l(+)-Alanine and d(-)-Alanine in Water at 25.0°*  
(Authors' Data)

Composition of mixture			Solubility of mixture per 100 gm. water					
<i>l</i> (+)-Alanine	<i>d</i> (-)-Alanine	Water	Experimental		Theoretical (no <i>dl</i> ) (a)	Difference between experimental and theoretical (a)	Theoretical ( <i>dl</i> ) (b)	Difference between experimental and theoretical (b)
			Individual	Average				
gm.	gm.	gm.	gm.	gm.	gm.	per cent	gm.	per cent
0.0163	1.6172	8.93	16.793 16.643 16.837*	16.72	16.68	0.24	16.87	0.89
0.0193	1.9112	8.93	16.705 16.757 17.010*	16.73	16.72	0.06	16.93	1.2
0.0817	1.5518	8.93	17.416 17.403 17.477	17.43	17.42	0.06	18.33	5.0
0.0965	1.8340	8.93	17.471 17.428 17.450	17.45	17.58	0.74	18.66	6.7
1.6172	0.0163	8.93	16.669 16.582 16.816	16.69	16.68	0.06	16.87	1.1
1.9112	0.0193	8.93	16.662 17.006* 16.758	16.71	16.72	0.06	16.93	1.3
1.5518	0.0817	8.93	17.343 17.440 17.358	17.38	17.42	0.23	18.33	5.3
1.8340	0.0965	8.93	17.522 17.505 17.537	17.52	17.58	0.34	18.66	6.3

\* Omitted in calculation of the average value.

Schmidt (34), *dl*-alanine exists as a racemic compound in the crystalline state as well as in aqueous solutions of the crystalline material.

From a consideration of all of the foregoing observations it may be concluded that the authors' alanine antipodes had identical specific rotations and solubilities and that they were of high purity.

*Evaluation of Specific Rotations of l(+)-Alanine and d(-)-Alanine Reported in Literature*

The specific rotations of l(+)-alanine and d(-)-alanine reported in the literature and the authors' values for these antipodes are given in Table V.

TABLE V  
*Specific Rotations of l(+)-Alanine and d(-)-Alanine\* (from Literature)*

	c (solute per 100 cc. solution)	Solvent	Polarimetric tube length	Temperature	$\alpha$ (observed optical rotation)	$[\alpha]_D^{25}$ (specific rotation at observed temperature)		Bibliographic reference No
						Literature	Present authors	
	gm.		dm.	°C.	degrees	degrees	degrees	
l(+)-Alanine	5.790	0.97 N HCl	2	15	+1.70	+14.7	†	36
	8.390	0.93 " "	2	15	+2.40	+14.3†	+14.1	36
	1.781	3 N HCl	2	20		+9.7	†	35
	1.781	0.2 N HCl	2	20		+7.3	†	35
	6.09	0.68 " "	1	20	+0.82	+13.5†	+13.6	6
	6.25§	0.70 " "	1	20	+0.84	+13.5†	+13.6	13
	6.44	0.72 " "	1	20	+0.85	+13.2	+13.6	6
	7.40	0.83 " "	1	20	+1.07	+10.3	+13.7	38
	8.27	1 N HCl	1	20	+0.97	+11.7	+13.7	39
	1.781	0.1 N HCl	2	20		+3.9	†	35
	1.781	0.02 " "	2	20		+1.9	†	35
	5.99	6 N HCl	2	25	+1.64	+13.7	+14.1	40
	1.225§	6 " "	2	30.6	+0.295	+12.0	+13.2	
	6.360	0.72 N HCl	1	20	+0.87	+13.7†	+13.8	12
	3.070§	1 N HCl	1	20	+0.63	+14.6	†	20
	10.3	Water	2	22	+0.55	+2.7	+2.58	12
d(-)-Alanine	6.780§	0.76 N HCl	2	20	-1.85	-13.65†	-13.62	13
	7.240§	0.81 " "	2	20	-2.10	-14.52	-13.65	38
	1.344§	6 N HCl	2	30.4	-0.392	-14.6	-13.3	
	8.721§	0.88 N HCl	1	20	-0.68	-9.50	†	16
	3.130§	1 N HCl	2	20	-1.28	-14.5	†	20
	6.53§	0.83 N HCl	2	20	-1.81	-13.63†	-13.60	14
	8.8§	Water	1	20 (?)	-0.21	-2.38	-2.44	13

\* Specific rotations given for l(+)-alanine in alkaline solutions by Lutz and Jirgensons (35) and in acid and water at 5461 Å. by Clough (36) and Barrow and Ferguson (37) were not considered.

† The specific rotation could not be determined with satisfactory accuracy from the present authors' data.

‡ These values and those of the present authors given in the adjoining column differ by an amount within, or slightly beyond, the probable experimental error.

§ Antipode obtained by resolution. All other forms were isolated from protein sources.

|| Dunn, M. S., Butler, A. W., and Naiditch, M. J., unpublished data.

The latter were determined by means of the factors (given in Tables VI and VII) which were derived by the interpolation of families of curves drawn from plots of the authors' experimental data given in Table II. These estimated specific rotations are considered to be accurate with a probable error of less than 1 per cent.

It may be noted that only six of the twenty-three values from the literature coincide within, or slightly beyond, the probable experimental error, with the specific rotations established by the present authors. It is significant, also, that the remaining values from the literature could not be evaluated

TABLE VI  
*Influence of Temperature on  $[\alpha]_D$  of *l*(+)-Alanine and *d*(-)-Alanine*

Solvent	Moles acid per mole amino acid	$\rho$ (solute per 100 gm solution)	Temperature range	Temperature coefficient				
				15°	20°	25°	30°	40°
		gm.	°C					
4.82 N HCl	21	2	0-45	-0.073	-0.071	-0.069	-0.066	-0.062
5.97 " "	26	2-10	25			-0.069		
5.97 " "	15-5	3.5-10	0-45	-0.073	-0.071	-0.069	-0.066	-0.062
7.25 " "	32	2	0-45	-0.073	-0.071	-0.069	-0.066	-0.062
0.228-0.884 N HCl	1	2-7.5	0-45	-0.063	-0.060	-0.057	-0.055	-0.054
Water	0	6-10	0-45	-0.040	-0.040	-0.040	-0.040	-0.040

TABLE VII  
*Influence of Solute Concentration on  $[\alpha]_D$  of *l*(+)-Alanine and *d*(-)-Alanine*

Solvent	Temperature range	$\frac{\Delta[\alpha]_D^*}{\Delta c}$	Concentration range ( $\rho$ )
	°C		
Water	0-45	0.13	6-10
6 N HCl	0-45	0.09	3.5-10
6 " "	25	0.09	2-10

\* Change in specific rotation per gm. change in concentration of solute.

with satisfactory accuracy, because it is extremely difficult to measure rotations with high precision at low concentrations of solute and acid. In these experimental regions, the rotations change markedly with minute changes in concentration of solute and acid.

It is considered to be of the utmost importance that measurements of optical rotation of amino acids be made under conditions such that the calculated specific rotations will have at least three significant figures. Otherwise, optical rotations are practically valueless as a criterion by which the degree of purity of an amino acid may be determined.

It is desirable to mention here that Levy and Palmer (21) have called attention to a misinterpretation by Dunn (41) of the specific rotation data of Pacsu and Mullen (20). In this statement, "Pascu and Mullen give  $[\alpha]_D^{20} = +10.33^\circ$  for *l*(+)-alanine and  $-10.30^\circ$  for *d*(-)-alanine in 1 *N* HCl," it was assumed that these values referred to the specific rotation of the "free base" alanines in 1 *N* HCl. In reality, these values are those calculated for the alanine hydrochlorides. Because their rotations for the alanine antipodes were numerically larger than the values reported by Fischer (13), Pacsu and Mullen concluded that "the separation of the two forms was more perfect in the method now given" and it was inferred that they had prepared the alanines in a state of purity higher than that of Fischer's products. The present authors were not able to corroborate this view. Instead, they give evidence in the present paper which appears to prove the high purity of both of Fischer's alanines. Furthermore, it seems true from the data in Table V that the specific rotations reported by Pacsu and Mullen should have been smaller than those found by Fischer. These authors stated that their values were obtained "under similar conditions" to those of Fischer and that, "Concentration apparently has no significant effect on the specific rotation of alanine itself." It may be noted, however, that the concentrations of solute per 100 cc. of solvent employed by Fischer were more than twice those of Pacsu and Mullen, while the present authors have shown that specific rotations of the alanines decrease in magnitude significantly with decreases in concentration of solute.

It is not possible to evaluate the reliability of the specific rotations reported by Levy and Palmer, inasmuch as these authors failed to stipulate some of the essential conditions.

#### SUMMARY

*l*(+)-Alanine and *d*(-)-alanine have been prepared in a state of high purity by resolution of *dl*-alanine and recrystallization of the antipodes. It has been shown that these isomers had identical specific rotations and solubilities within the limits of small percentage errors. The specific rotations of the alanine antipodes given in the literature have been evaluated by means of temperature and solute concentration factors derived from the present authors' data.

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# PREPARATION OF SHEEP PITUITARY GONADOTROPIN AND RECOVERY OF THE LACTOGENIC HORMONE\*

By W. H. McSHAN AND ROLAND K. MEYER

(From the Department of Zoology, University of Wisconsin, Madison)

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In various methods that have been reported for the preparation of pituitary gonadotropin, such as those of Jensen, Simpson, Folksdorf, and Evans (1) and Chow, van Dyke, and Greep (2), the active material is obtained either by precipitation with organic liquids or by salt precipitation, after which the gonadotropin or gonadotropins are separated from the precipitate. However, Fevold, Lee, Hisaw, and Cohn (3) fractionated an aqueous extract of fresh sheep pituitary glands by dialysis with ammonium sulfate solution, and Cartland and Nelson (4) purified the gonadotropin of pregnant mare serum by precipitating the inert material with acetone which left the hormone in solution.

This report is concerned with the presentation of a method for the purification of sheep pituitary gonadotropin which is similar to the latter two methods in that inert material is separated from an aqueous extract by the use of buffers during dialysis. Subsequently the major part of the gonadotropin is recovered from the solution in concentrated form. The final product is free of lactogenic hormone, and the greater part of the latter activity can be recovered by alcoholic extraction of the residue. The biological assay and the solid, carbohydrate, nitrogen, and protein contents of a number of preparations obtained by this method are also given.

## EXPERIMENTAL

A stock solution of acetate buffer of pH 4, M in sodium acetate and 4.1 M in acetic acid, was diluted 1:14, and 0.14 M dibasic sodium phosphate solution was diluted 1:6 and 1:12 for dialytic purposes. Cellophane tubing, 28 mm. in diameter, for use in dialysis was obtained from E. H. Sargent and Company. Whole sheep pituitary powder obtained from The Wilson Laboratories and acetone-dried anterior lobes of sheep were used. The quantities of materials given in the following procedure are for use with 100 gm. of pituitary powder, but the method has been used successfully with 500 and 600 gm. lots.

*Method of Purification*—Sheep pituitary powder was mixed with water in the ratio of 1 gm. to 10 ml., cooled, and shaken for 10 hours. 2 ml. of

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toluene were added as preservative. The mixture was centrifuged and the residue was extracted a second time in the same way. The two extracts were combined to form Fraction A. Residue L contained the major part of the lactogenic activity and was retained.

Fraction A was adjusted to pH 5 by dialysis of one-fourth of its volume against dilute acetate buffer of pH 4 for 30 minutes, at which time the dialyzed part was mixed with the three-fourths that was not dialyzed. Usually the dialysis had to be repeated for two shorter periods of time in order to complete the adjustment of the solution to pH 5.

The precipitate that formed at pH 5 was recovered by centrifuging. It was washed with 100 ml. of water by shaking with glass beads, and centrifuged to give Fraction R. The clear supernatant liquid was added to Fraction B, soluble at pH 5.

Fraction B was adjusted to pH 4 by dialysis against dilute acetate buffer, and the precipitate was recovered from the soluble Fraction C by centrifuging. It was suspended in 75 ml. of distilled water and dissolved by dialysis against 1:6 phosphate solution, after which it was again adjusted to pH 4 by dialysis against the acetate buffer. The precipitate that formed was recovered by centrifuging and reprecipitated twice, after which it was again dissolved and designated as Fraction P. The three soluble fractions obtained at pH 4 were added to the soluble Fraction C.

Fraction C was dialyzed against distilled water for 24 hours, during which time four changes of water were made. A light precipitate formed which was recovered by centrifuging and designated as Fraction D.

The dialyzed soluble fraction was treated with 4.5 volumes of cold 95 per cent ethyl alcohol, and cooled overnight. A fine precipitate formed which was recovered by centrifuging, or, if there were large quantities, by supercentrifugation. It was dissolved in 40 ml. of water by dialysis against 1:12 phosphate solution, after which it was adjusted to pH 4 by dialysis against dilute acetate buffer diluted 1:1 and then dialyzed against three changes of distilled water and centrifuged. If any precipitate was obtained, it was dissolved in 10 ml. of water and reprecipitated as above. The resulting supernatant liquid was added to the original and the mixture then dialyzed against 1:12 phosphate solution. This solution contained the gonadotropin, which was recovered in the dry form by the lyophile process.

The adjustment of Fraction A to pH 5 and the resulting soluble Fraction B to pH 4 can be accomplished by dialysis against 0.03 N HCl. However, this alteration in the above procedure was not studied in detail.

The nitrogen content of the preparations was determined by a modification of the micromethod of Johnson (5), and the carbohydrate by the carbazole method of Gurin and Hood (6). The nitrogen values were multiplied by 6.25 to obtain the protein values.

*Recovery of Lactogenic Hormone*—The major part of the lactogenic hormone of the original pituitary powder was recovered from Residue L of Fraction A by the method of Bates and Riddle (7) except that extractions were made at pH 10 with 75, 78, or 80 per cent ethyl alcohol in place of 70 per cent.

The lactogenic hormone was extracted also from pituitary powder by this procedure before the gonadotropin was removed by aqueous extraction. A great part of the gonadotropin was recovered in the crude form by aqueous extraction of the residue that remained after the alcoholic extraction. However, it was not possible to purify readily the gonadotropin of this extract by the method outlined above.

*Assay Procedures*—The preparations and side fractions were assayed in 21 day-old female rats. The total amount of material was dissolved in 4.5 ml. of water and given in nine injections of 0.5 ml. each beginning on the afternoon of the 1st day and followed by morning and afternoon injections the next 4 days. Autopsy was performed during the morning of the 6th day. The ovaries were removed, examined for the presence of follicles and corpora lutea, and weighed.

The same procedure was used in testing the fractions for luteinizing activity. If a particular fraction did not stimulate the ovaries of immature rats when given alone but produced corpora lutea when given in combination with follicle-stimulating hormone, it was considered to contain luteinizing activity. The method of Evans *et al.* (8) was used in testing the fractions for inhibitor.

Assay of the fractions for lactogenic hormone was carried out by making shallow injections into the pectoral muscles of adult pigeons twice daily for 4 days. The birds were killed on the 5th day, the crop glands were removed immediately and weighed, and the degree of stimulation noted.

#### DISCUSSION

*Activity of Different Fractions*—The increase in the weight of the ovaries from the normal value of 13 mg. to the average weights given in Table I serves as a quantitative measure of the gonadotropic activity of the fractions obtained at each step in the procedure. Fraction A, the soluble Fractions B and C, and Fraction F were tested, each in a dose of 100 mg.<sup>1</sup> The ovaries produced by each of these fractions had an average weight of 111, 105, 111, and 113 mg. respectively. These results show that there was little difference in the activity of these fractions and that there was little activity lost during the fractionation process. However, Fractions R and P did contain some luteinizing hormone, but there was ample luteinizing activity left in the purified preparation to augment the action of the follicle-stimulating

<sup>1</sup> All doses are given in mg. equivalents of pituitary powder.

hormone and produce corpora lutea. Furthermore, considerable inhibitor found in Fractions R and P was separated from Fraction F, and this may account in part for the high activity of the purified preparation.

The presence of luteinizing activity in Fraction R was shown by the fact that doses of 500 mg. and 1 gm. gave ovaries having an average weight of 19 and 22 mg. respectively, while, when 500 mg. were given with 500 mg. of

TABLE I  
*Gonadotropic Content of Fractions Obtained during Purification*

Fraction*	Kind of fraction	Total dose	No of rats	Ovaries	
				Average weight	Qualitative response
		mg †		mg	
A	Aqueous extract	100	23	111	Many corpora lutea
B	Portion of A soluble at pH 5	100	12	105	" " "
C	Portion of B soluble at pH 4	100	19	111	" " "
F	Purified	100	47	113	Few " "
R	Portion of A insoluble at pH 5	500	15	19	
"	" "	1000	3	22	
FSH	FSH50689	500	24	58	Only follicles
R + FSH	Portion of A insoluble at pH 5	500 each	8	116	Many corpora lutea
P	Portion of B insoluble at pH 4	500	13	18	
"	" "	1000	10	24	
" + FSH	" "	500 each	16	102	Many corpora lutea
D	Insoluble portion of C dialyzed at pH 4	100	9	16	
"	" "	200	3	14	
"	" "	500	15	61	Few corpora lutea
"	" "	1000	3	79	" " "

\* Fractions A, B, and C from one fractionation had a non-dialyzable solid content of 88.8, 70.9, and 24 mg. respectively per gm. equivalent of pituitary powder. The average value for the protein content of nine F fractions was 4.25 mg. per gm.

† Indicates mg. equivalents of pituitary powder.

follicle-stimulating hormone, ovaries containing many corpora lutea and having an average weight of 116 mg. were obtained. Similarly the data show that Fraction P was essentially inactive when injected alone and that it produced corpora lutea when given with follicle-stimulating hormone.

Fractions D showed some variation in activity but on the average contained only a relatively small amount of activity,\* as no stimulation was

produced by 200 mg., while 500 mg. produced ovaries that had an average weight of 61 mg.

*Purified Preparations*—The assays of the individual purified preparations together with the carbohydrate expressed as glucose, the dry weight, and the nitrogen and the protein content of 100 mg. equivalents of original pituitary powder are given in Table II. This method of expressing the results shows the amounts of each of these substances contained in the total dose of hormone required to produce ovaries having the average weights given in Table II. These results show that the purified preparations are high in ac-

TABLE II  
*Assay and Analysis of Purified Fractions*

Fraction No	Total dose	Analyses in terms of total dose				No of rats	Average weight of ovaries
		Dry weight	Nitrogen	Protein	Carbohydrate as glucose		
	mg *	mg	γ	mg	mg		mg
F55†	100	0.44	50.3	0.31	0.019	5	65
"	125					6	80
"	200					6	134
F56	100	0.76	96.7	0.60	0.087	6	126
"	50					6	62
F58†	100	0.65	67.4	0.42	0.036	9	58
"	200					3	138
F68	100	0.77	67.7	0.42	0.106	8	121
F71	100	0.90	68.0	0.42	0.057	6	94
F72	100	0.84	46.4	0.29	0.062	9	107
F73	100	1.00	59.4	0.37	0.062	6	108
F74	100	1.15	94.3	0.59	0.098	6	127
F75	100	0.52	66.3	0.41	0.044	6	106

\* Indicates mg. equivalents of pituitary powder.

† Made from whole pituitary powder.

tivity and they contain on the average only  $0.425 \pm 0.032$  per cent protein in terms of the weight of the original pituitary powder.

The average ovarian weights produced by 100 mg. of Fractions F55 and F58 which were prepared from whole sheep pituitary powder were smaller than those produced by the same dose of the other preparations that were made from anterior lobe powder. These two preparations also had a smaller carbohydrate content than any of the other preparations.

Fractions F71, F72, F73, and F75 were similar in activity, and their protein and carbohydrate contents were constant. Fractions F56, F68, and F74 produced ovaries that had an average weight of 126, 121, and 127 mg., respectively, and were also higher in protein and carbohydrate.

The purified gonadotropic preparation is highly soluble in water or buffer solution, as shown by the fact that 20 gm. equivalents or 131 mg. of Fraction F76 with the exception of 2 mg. dissolved in sufficient buffer to give 1 ml. of solution. The small amount of insoluble material was washed, dried, and tested in the immature rat. It contained little, if any, activity, as 10 gm. equivalents produced ovaries that had an average weight of only 14 mg.

The preparations contain only very small amounts of serum proteins as compared to unpurified preparations, as shown by the precipitin reaction. They are antigenic, however, in the sense that antigonadotropic factor is produced in the blood of rabbits on injection of the preparations daily for 3 weeks.

The method can be repeated with consistent results, as shown by the fact

TABLE III  
*Assay of Fractions for Gonadotropic and Lactogenic Activities*

Fraction	Assay						
	Rat			Pigeon			
	Total dose	No of rats	Weight of ovaries	Total dose	No of birds	Crop glands	
						Average weight	Qualitative response
	mg *		mg.	mg.*		gm	
A	100	23	111	100	3	1.49	Not stimulated
				200	3	1.48	" "
				500	8	3.16	Stimulated
F122	100	6	110	500	3	1.69	Not stimulated
LR201	200	4	13	100	6	4.00	Stimulated
	500	3	16	200	4	5.58	"

\* Indicates mg. equivalents of pituitary powder.

that it has been used in making more than twenty preparations. It is important to point out, however, that this method is not satisfactory if Fraction A, B, or C is precipitated with acetone at any stage of the procedure. The concentration of the activity with ethyl alcohol is the only case in which an organic liquid is used.

*Separation of Lactogenic Hormone*—Fraction A from which the gonadotropic preparation was made did not show lactogenic activity in doses of 100 and 200 mg. but 500 mg. stimulated the crop glands of pigeons as shown in Table III. However, the crop glands were not stimulated by 500 mg. of the purified gonadotropic preparation, Fraction F122. These results demonstrate that the small amount of lactogenic activity contained in the

crude Fraction A was separated from the gonadotropin during the fractionation process.

The major part of the lactogenic hormone activity of the pituitary powder was recovered from the residue of Fraction A by extracting it with 75 per cent ethyl alcohol. One of these lactogenic preparations, Fraction LR201, had a solid content of 32 mg. per gm. of original pituitary powder and was relatively free of gonadotropic activity, as shown by the data of Table III. The solid content of these preparations was greater when 78 or 80 per cent alcohol was used.

Extraction of anterior lobe powder first with 75, 78, or 80 per cent alcohol at pH 10 removed the major part of the lactogenic activity with little if any gonadotropic activity. Under these conditions the solid content of the lactogenic fraction was low. When the residue from the alcoholic extraction was extracted with water, a great part of the gonadotropic activity was recovered in the crude form, but the gonadotropin of this extract is not readily purified.

These results indicate further that sheep pituitary gonadotropin is soluble in water and is extracted by it, but not by 75 to 80 per cent aqueous solutions of alcohol at pH 10, while the reverse is true for the lactogenic hormone. In this connection Fleischer (9) reported recently that the lactogenic hormone of fresh pituitary glands in the presence of the proper concentration of acid is soluble in 95 per cent ethyl and 99.8 per cent methyl alcohol. Under these conditions considerable inert protein was removed along with the lactogenic activity, while, as stated above, extraction of sheep pituitary powder with 75 to 80 per cent alcohol at pH 10 removes the hormone with a relatively small amount of inert material.

#### SUMMARY

A method consisting of simple procedures is presented for the preparation of sheep pituitary gonadotropin by the elimination of inert material from an aqueous solution of the hormone. The major part of the gonadotropic activity is concentrated in the final product, which has a protein content of approximately 0.4 per cent of the weight of the original pituitary powder. The assays and the nitrogen, protein, and carbohydrate content of nine purified preparations are given.

The gonadotropin is obtained free of lactogenic hormone, while the greater part of the latter activity can be recovered from the residue by extraction with aqueous alcohol.

The side fractions were relatively inactive, although two of these fractions contained some luteinizing activity, while a third stimulated the ovaries of immature rats when given in large doses.



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# THE INFLUENCE OF PREVIOUS DIET ON THE PREFERENTIAL UTILIZATION OF FOODSTUFFS\*

## I. FASTING KETOSIS AND NITROGEN EXCRETION AS RELATED TO THE FAT CONTENT OF THE PRECEDING DIET

BY SIDNEY ROBERTS AND LEO T. SAMUELS

*(From the Division of Physiological Chemistry, Department of Physiology, University of Minnesota Medical School, Minneapolis)*

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Contradictory evidence has been presented with regard to the influence of the fat content of the diet and the amount of liver fat on the degree of fasting ketosis. The studies of Deuel and his coworkers seemed to indicate that these phenomena were interrelated (1). However, as MacKay later pointed out (2, 3), subnormal amounts of the high fat diets were consumed, so that protein intake was also lowered. This was reflected in a reduction in nitrogen excretion in fasting animals previously fed high fat diets (1, 3). MacKay concluded that the degree of fasting ketosis was related only to the protein of the preceding diet.

In the present series of investigations, we have applied to this problem the technique of feeding by stomach tube described by Reinecke, Ball, and Samuels (4). By this means, the fat content alone of the diet can be varied. We have been able to show that the degree of fasting ketosis is influenced by the fat content of the preceding diet. This is true even though protein intake was adequate and constant, and nitrogen excretion was similar in all groups of experimental animals.

### *Materials and Methods*

Adult male rats, obtained from Sprague-Dawley, Inc., and weighing 250 to 300 gm., were divided into two groups having similar weight ranges. All animals were first fed a mixed diet by stomach tube, composed of equal parts of the high fat and high carbohydrate diets described in an earlier report (5).

After 4 days on this régime, by which time the animals had become accustomed to this method of feeding, one group was transferred to the high fat diet, the other to the diet high in carbohydrate. The high fat diet was composed of 85 per cent corn oil (on a caloric basis) and 15 per cent lactal-

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bumin; the high carbohydrate diet of 83 per cent dextrin, 2 per cent corn oil, and 15 per cent lactalbumin. Both diets were supplemented with adequate quantities of vitamins and minerals (5). Percentage absorption was probably quite similar in the two groups (6).

These semiliquid diets were fed twice daily in equicaloric amounts (equal volumes) for a period of 6 weeks. At the end of this time, all animals were placed in metabolism cages over funnels for the collection of urine, and feeding was discontinued. Free access to a 1 per cent salt solution was allowed, to promote adequate diuresis. Urine collections were made under toluene for the first and second 18 hours after the deprivation of food.

The urine filtrates were analyzed for total acetone bodies by the Van

TABLE I

*Nitrogen and Acetone Body Excretion of Rats during Early Fasting after 6 Weeks on High Fat and High Carbohydrate Diets Fed by Stomach Tube*

All values are expressed as mg. per sq. dm. of body surface per 18 hours.

High carbohydrate				High fat			
0-18 hrs		18-36 hrs		0-18 hrs		18-36 hrs	
Acetone bodies	Nitrogen	Acetone bodies	Nitrogen	Acetone bodies	Nitrogen	Acetone bodies	Nitrogen
0.2	16.6	0.8	9.1	8.9	19.5	24.0*	9.7
0.2	20.8	0.4	19.5	5.9	20.8	2.0	12.5
0.1	17.8	0.8	13.0	7.2	21.6	4.6	11.2
0.1	21.1	0.6	17.4	10.0	18.1	2.8	13.3
0.3	11.2	0.8	12.6	8.1	20.3	1.2	11.5
				3.4	15.2	1.2	8.0
				2.4	20.0	1.8	11.0

\* This animal may have become diabetic. It also exhibited a high blood sugar (150 to 175 mg. per cent). However, urinary glucose was negligible at this time.

Slyke procedure (7); nitrogen was determined by the Kjeldahl method, the boric acid modification being employed (8).

In another experiment, 200 gm. rats of the same strain were maintained on the tube-fed diets for a period of 3 weeks. At the end of this time, they were fasted 24 hours, and 5 cc. of aortal blood were drawn under amytal anesthesia. The animals were then sacrificed and liver weights obtained. Total blood acetone bodies were determined by the procedure of Van Slyke and Fitz (9).

### Results

Table I clearly reveals the existence of a significant urinary ketosis during the first 36 hours of fasting following 6 weeks on a high fat diet. This

ketosis is evidently absent in similar animals previously maintained on the diet high in carbohydrate. Nitrogen excretion during this same period of time is uninfluenced by the fat content of the preceding diet.

That this ketosis also extends to the blood is shown in Table II. Animals forcibly fed a high fat diet for 3 weeks and then fasted 24 hours exhibited a high level of blood acetone bodies. No determinable quantities were present in the blood of carbohydrate-fed animals. Livers weighed more in the fat-fed group (Table II), and appeared grossly fatty.

In both experiments, the range and average of the body weights were quite similar for fat-fed and carbohydrate-fed animals.

TABLE II

*Blood Acetone Bodies and Liver Weights of Rats Previously Forcibly Fed High Fat and High Carbohydrate Diets for 3 Weeks and Then Fasted 24 Hours*

High carbohydrate		High fat	
Blood acetone bodies	Liver weight per 100 gm body weight	Blood acetone bodies	Liver weight per 100 gm. body weight
<i>mg. acetone per 100 cc.</i>	<i>gm.</i>	<i>mg. acetone per 100 cc.</i>	<i>gm.</i>
<1	2.67	8.8	3.04
<1	2.74	9.4	3.46
<1	2.74	6.7	3.40
<1	2.56	7.8	2.78
<1	2.68	6.1	2.65
<1	2.67	8.1	3.02
<1	2.73	7.3	3.17
<1	2.72	7.6	2.98
	2.72		2.83
			3.54

## DISCUSSION

The results reported above show clearly that the degree of fasting ketosis varies with the fat content of the preceding diet as well as with the protein content. In fat-fed animals, the significant blood and urinary ketosis was associated with a large liver, which was probably fatty (10). Body weights were quite comparable in the animals fed high fat and high carbohydrate diets, as was also absorption of ingested food (6).

Protein metabolism is apparently unaffected by the fat content of the diet, at least during subsequent fasting. Thus nitrogen excretion was the same in both groups of experimental animals.

Animals previously maintained on a high fat régime have been observed to spare carbohydrate in the earlier stages of fasting. The opposite situation holds for carbohydrate-fed animals (5, 6, 10). The present investiga-

tion indicates that this phenomenon of "preferential utilization" is due to an increased combustion of fat in the former group. Thus liver and muscle glycogen is used much more rapidly in the carbohydrate-fed group during early fasting (6, 10). The presence of acetone bodies in the blood and urine of the group fed the high fat diet is apparently an index of accelerated fat metabolism. This conclusion cannot be avoided in view of the fact that the basal metabolic rates of the two groups are the same (11), as are also the rates of glyconeogenesis from protein (nitrogen excretion data).

Since acetone bodies are apparently being used at a faster rate in those animals in which glucose utilization is probably depressed, the classical ketolysis theory of Shaffer becomes untenable (12).

Similarly, strict antiketogenesis cannot hold, since acetone body production was occurring at a rapid rate in the fat-fed animals at a time when liver glycogen was not exhausted, and was, in all likelihood, higher than in the carbohydrate-fed group (10).

It seems probable that the phenomenon of fasting ketosis can be considered without direct regard to the utilization of glucose. Whenever fat metabolism is accelerated, as by fat feeding or by fasting, the production and utilization of acetone bodies may very well be increased. Fasting ketosis then, as related to the nature of the preceding diet, is a reflection of the phenomenon of preferential utilization. From this point of view, the fasting ketosis noted after maintenance on a low protein diet may be considered to be due to a compensatory acceleration of fat metabolism, beginning during feeding and continuing over into the early fasting period.

#### SUMMARY

Adult male rats forcibly fed a high fat diet for 3 to 6 weeks exhibited a significant early fasting ketosis. This was absent in animals similarly treated but fed a high carbohydrate diet. Protein intake and nitrogen excretion during fasting were the same in both groups.

The ketosis was evidenced by high levels of blood and urinary acetone bodies and large, grossly fatty livers in the fat-fed animals.

Evidence is presented that fasting ketosis is a reflection of accelerated fat metabolism initiated during feeding and continuing after the withdrawal of food. This is the phenomenon of preferential utilization.

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# STUDIES ON THE MECHANISM OF DEAMINATION OF SERINE AND THREONINE IN BIOLOGICAL SYSTEMS\*

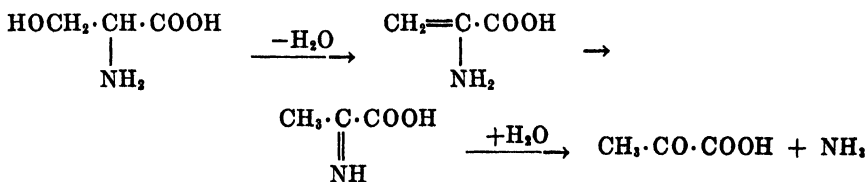
BY ERWIN CHARGAFF AND DAVID B. SPRINSON

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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The biological fate of serine is of particular interest, since this hydroxy-amino acid may be considered to form, in certain respects, a connecting link between the metabolism of proteins and that of phospholipids. In a recent brief communication from this laboratory (1) the mechanism of the deamination of serine by *Bacterium coli* was discussed. The present paper extends the experiments to the essential amino acid threonine and offers a description of the experimental results obtained with both serine and threonine and the following bacterial species: *Bacterium coli*, *Clostridium welchii*, *Pseudomonas pyocyanea*, *Proteus* OX-19. A few orienting experiments with mammalian tissue are likewise included, but their detailed presentation will be reserved for a later publication.

With resting suspensions of *Bacterium coli*, which were very active in the deamination of both *d*- and *l*-serine (2), *dl*-O-ethylserine, *l*-phosphoserine, and phosphatidyl serine from beef brain were not deaminated; *dl*-O-methylserine was attacked very slowly but, in contrast to serine, under aerobic conditions only. The inhibition of the deamination of serine on replacement of the hydroxylic hydrogen atom suggests that the first step in the reaction involves the removal of the elements of water.



Threonine would similarly be deaminated to  $\alpha$ -ketobutyric acid.

It is obvious that if this formulation is correct, the fundamental equation for the oxidative deamination of amino acids (3-6), viz.  $\text{R}\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH} + \frac{1}{2}\text{O}_2 = \text{R}\cdot\text{CO}\cdot\text{COOH} + \text{NH}_3$ , would, in this special case, have to be replaced by  $\text{R}\cdot\text{CH}(\text{OH})\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH} = \text{R}\cdot\text{CH}_2\cdot\text{CO}\cdot\text{COOH} + \text{NH}_3$ .

It is possible under suitable conditions, with all the bacterial species examined, to isolate *pyruvic acid* and  $\alpha$ -ketobutyric acid in considerable

\* This work has been supported by a grant from the John and Mary R. Markle Foundation.



amounts in the form of their 2,4-dinitrophenylhydrazones as the deamination products of serine and threonine respectively. The postulate that the pathway of breakdown of these hydroxyamino acids differs from that of the other amino acids is further strengthened by the finding (2, 7), confirmed in this laboratory, that serine is deaminated by *Bacterium coli* under aerobic and anaerobic conditions, whereas alanine is attacked only aerobically.<sup>1</sup> The experiments here discussed have, furthermore, generally shown that both serine and threonine are deaminated anaerobically by all bacterial species examined. Both *d*- and *l*-serine were attacked, a finding already reported for *Proteus* (9) and *Pseudomonas pyocyanea* (10) by Bernheim and his collaborators.

A discussion of the transformations which the hydroxyamino acids undergo in the animal organism will be deferred to a later occasion. It will suffice to point out here that in the course of the present study cell-free extracts from rat, mouse, and rabbit livers were obtained which, especially after the addition of  $Mg^{++}$  to the thoroughly dialyzed solutions, attacked serine with liberation of appreciable amounts of ammonia under anaerobic conditions. Like the bacterial preparations discussed above, these extracts failed to deaminate the O-ethers of serine. The anaerobic formation of pyruvic acid from serine by a rat liver extract was demonstrated by the isolation of the corresponding 2,4-dinitrophenylhydrazone. In contrast, kidney slices have been found not to deaminate serine under anaerobic conditions (5).

The formation of  $\alpha$ -keto acids from serine and threonine is analogous to the conversion of serine to pyruvic acid by the action of hot acidic (11, 12) or alkaline (13) solutions or of acetic anhydride (14). The possible intermediate rôle of  $\alpha$ -aminoacrylic acid in the production of pyruvic acid from serine has been repeatedly discussed by Bergmann and his collaborators (15, 16) on the basis of *in vitro* experiments with glycylserine and alanylserine.<sup>2</sup> The findings on the breakdown of serine in biological systems here presented are in harmony with the views of Brand and his collaborators (19) and of Binkley and du Vigneaud (20) on the rôle of serine (or aminoacrylic acid) and its peptides as biological precursors of cysteine by way of cystathionine.

The conception of a dehydration as the primary step of attack on serine and threonine places the enzyme or enzymes performing this task in the class of dehydrases.<sup>3</sup> The most thoroughly studied representative of this group

<sup>1</sup> A review of the entire field of the bacterial breakdown of amino acids will be found in an article by Gale (8).

<sup>2</sup> Compare also the discussions by Dakin (17) and Nicolet (18) of  $\alpha,\beta$ -unsaturated  $\alpha$ -amino acids as hypothetical biological intermediates.

<sup>3</sup> This term is not to be confused with "dehydrogenases" (*cf.* (21)).

is enolase, which catalyzes the conversion of 2-phosphoglyceric acid into phosphopyruvic acid (22, 23). This enzyme is known to occur in *Bacterium coli* (24). We plan to compare the properties of the serine and threonine dehydrases of bacteria and animal tissues with those of other dehydrases and to define more sharply the substrate specificities of the enzymes in question.<sup>4</sup>

## EXPERIMENTAL

### Methods

**Substrates**—*Phosphatidyl serine* was prepared from beef brain (26). The preparation used had the following analytical figures: P 3.57, N 1.69, amino N 1.67, amino acid N 1.37, iodine value 72.

A sample of *l-phosphoserine* was obtained through the courtesy of Dr. F. Lipmann of the Massachusetts General Hospital, Boston. *dl-O-Methylserine* was prepared from the corresponding N-benzoyl derivative kindly supplied by Dr. H. E. Carter of the University of Illinois, Urbana. The authors are indebted to Dr. V. du Vigneaud for the *dl-O-ethylserine* employed in these experiments. *dl-Serine* and *dl-threonine* were Merck products.

**Bacterial Suspensions**—*Bacterium coli*, *Pseudomonas pyocyanea*, and *Proteus* OX-19 were grown in Roux bottles at 37° on a medium composed of 2 per cent of Bacto-tryptone (Difco), 3 per cent of agar, 0.5 per cent of sodium chloride, and 0.5 per cent of yeast concentrate. For the anaerobic cultivation of *Clostridium welchii* a similar medium, but with the omission of agar, was employed. The microorganisms were, after 20 to 24 hours growth, collected in cold physiological saline, sedimented in a refrigerated angle centrifuge, and washed three times with ice-cold saline.

**Analytical Procedures**—For the determination of ammonia by the Nessler procedure, an equal volume of a solution of 12.4 gm. of  $H_3BO_3$  in 110 cc. of  $N NaOH$  (27) was used to make the solutions alkaline and 1 drop of *n*-decanol was added, the alkaline solutions were aerated, and the ammonia collected in 20 cc. of water containing 2 drops of 0.1  $N HCl$ . After the addition of the Nessler reagent, the solutions were brought to a volume of 25 cc. and the color intensities estimated by means of a Klett-Summerson photoelectric colorimeter. The values were ascertained from a standard curve obtained in the same manner.

The oxygen consumption was determined in the customary manner in Warburg vessels.

<sup>4</sup> The enzymatic degradation of cysteine with the formation of  $H_2S$ , ammonia, and pyruvic acid (25) exhibits many similarities with the reactions discussed here. The reversibility of the dehydration of  $\beta$ -hydroxy- $\alpha$ -amino acids, a characteristic of other dehydrases, has not been tested, as the corresponding unsaturated amino acids are not available.

A 0.6 to 0.8 per cent solution of 2,4-dinitrophenylhydrazine in 2 N HCl was employed for the precipitation of the keto acid hydrazones from the deproteinized solutions (28). Details will be found later in the paper.

### Deamination by Bacteria

*Attack on Various Substrates*—Representative data on the aerobic and anaerobic deamination of a number of substrates by several bacterial species are summarized in Table I. It will be seen that with *Bacterium coli dl*-

TABLE I

#### Deamination of Hydroxyamino Acids and Their Derivatives by Bacteria

The Warburg vessels in each experiment contained 1 cc. of the bacterial suspension (corresponding to a dry weight of 5 to 6 mg.), 1 cc. of 0.1 M phosphate buffer of pH 7.5, and 1 cc. of substrate solution containing 168  $\gamma$  of substrate N. The vessels were shaken in the thermostat at 38° in the presence of air or, in the anaerobic experiments, of O<sub>2</sub>-free nitrogen for the periods indicated in the first column. At the end of each experiment the vessel contents were immediately acidified. The figures have been corrected for NH<sub>3</sub> produced in the absence of substrate, as determined in control experiments.

Species	Substrate	Ammonia nitrogen	
		Aerobic	Anaerobic
<i>Bacterium coli</i> (1 hr.)	<i>dl</i> -Serine	127	66
	<i>l</i> -Serine	134	
	<i>dl</i> -Threonine	73	42
	<i>l</i> -Phosphoserine	0	
	Phosphatidyl serine	0	
	<i>dl</i> -O-Methylserine	23	0
	<i>dl</i> -O-Ethylserine	0	0
	<i>dl</i> -Alanine	114	0
<i>Pseudomonas pyocyanea</i> (90 min.)	<i>dl</i> -Serine	100	20
	<i>dl</i> -Threonine	107	75
	<i>dl</i> -Alanine	124	0
<i>Proteus</i> OX-19 (90 min.)	<i>dl</i> -Serine	125	97
	<i>dl</i> -Threonine	117	20

serine was deaminated as rapidly as was the *l* isomer. With the exception of O-methylserine which was attacked very slowly, but only under aerobic conditions, none of the O-substituted compounds was deaminated. *dl*-Alanine was attacked only under aerobic conditions, whereas the hydroxy-amino acids were deaminated anaerobically also, although at a slower rate.

Table II offers a comparison between oxygen consumption and ammonia production with serine and alanine as substrates. If pyruvic acid is the deamination product of both amino acids, the oxidative deamination of

alanine as compared with the breakdown of serine should manifestly require the uptake of 1 additional oxygen atom. A comparison of the  $O_2$ :  $NH_3$  ratios for serine and alanine given in Table II shows this to be approximately the case.

*Isolation of Keto Acids As Deamination Products*—Between 3 and 4 gm. of wet bacteria were suspended in water to give a total volume of 25 cc., and the suspension was shaken with 5 cc. of toluene for 5 to 10 minutes. Most of the toluene could be removed at this point, but this proved unnecessary. The bacterial suspension was added to a solution of the amino acid in 25 cc. of 0.1 M phosphate buffer of pH 7.5 and the mixture shaken in the presence of air or, in the case of *Clostridium welchii*, of nitrogen for 2½ hours at 38°. After centrifugation 6 to 10 cc. of 30 per cent trichloroacetic acid were added to the supernatant. The addition to the filtered solution of a

TABLE II

*Relationship between Oxygen Consumption and Ammonia Production with Serine and Alanine As Substrates*

The experimental arrangement is as in Table I.

Species	Substrate	Time	Oxygen consumed		NH <sub>3</sub> -N produced		$\frac{O_2}{NH_3}$
			min	c.mm	micro-moles	γ	micro-moles
<i>Bacterium coli</i>	dl-Serine	50	100	4.5	130	9.3	0.48:1
	dl-Alanine	50	179	8.0	110	7.9	1.0:1
	dl-Serine	90	248	11	122	8.7	1.3:1
	dl-Alanine	90	331	15	127	9.1	1.7:1
<i>Pseudomonas pyocyanea</i>	dl-Serine	90	149	6.7	100	7.1	0.94:1
	dl-Alanine	90	303	13.5	124	8.9	1.5:1

0.6 to 0.8 per cent solution of 2,4-dinitrophenylhydrazine in 2 N HCl produced the immediate precipitation of the hydrazone which, after being stored in the refrigerator overnight, was filtered off and twice recrystallized for analysis from ethyl acetate (in the case of the pyruvic acid hydrazone) or ethyl acetate-ligroin (for the α-ketobutyric acid hydrazone). The melting points of the crude products were only 2–3° lower than those of the purified samples. When dl-alanine was similarly treated in the presence of *Bacterium coli*, no hydrazone was precipitated. Since *Pseudomonas pyocyanea* decomposes α-ketobutyric acid only slowly, its hydrazone could be isolated from the deamination of threonine regardless of whether these bacteria had been previously treated with toluene or not. The results obtained with serine and threonine are presented in Tables III and IV respectively.

*Deamination by Cell-Free Liver Extracts*

*Preparation of Extracts*—Freshly obtained rat or mouse livers were chilled, homogenized in a small tissue grinder, and extracted with 2 volumes

TABLE III  
*Pyruvic Acid from dl-Serine*

Species	Substrate used	2,4-Dinitrophenylhydrazone					
		Yield		M p.*	Analysis†		
					C	H	N
	mg.	mg.	per cent of theoretical	°C.	per cent	per cent	per cent
<i>Bacterium coli</i> .	500	227	18	215	40.3	3.1	20.8
<i>Pseudomonas pyocyanea</i>	500	112	9	216	40.3	3.2	20.7
<i>Proteus</i> OX-19	500	255	20	215	40.3	3.0	20.7
<i>Clostridium welchii</i>	550	193	14	216	40.6	3.1	20.6

\* No depression of the melting point was observed on admixture of an authentic specimen of pyruvic acid 2,4-dinitrophenylhydrazone.

† Calculated for pyruvic acid 2,4-dinitrophenylhydrazone (268), C 40.3, H 3.0, N 20.9.

TABLE IV  
*α-Ketobutyric Acid from dl-Threonine*

Species	Substrate used	2,4-Dinitrophenylhydrazone					
		Yield		M p.*	Analysis†		
					C	H	N
	mg.	mg.	per cent of theoretical	°C.	per cent	per cent	per cent
<i>Bacterium coli</i>	500	340	29	195	42.6	3.6	19.8
<i>Pseudomonas pyocyanea</i> (with toluene)	500	456	39	195	42.5	3.7	19.7
<i>Pseudomonas pyocyanea</i> (without toluene)	500	354	30	195	42.8	3.7	19.6
<i>Proteus</i> OX-19	400	158	17	195	42.6	3.7	19.6

\* Mixed melting point with a sample kindly furnished by Dr. H. Waelsch of this College (cf. (29)), 195°.

† Calculated for α-ketobutyric acid 2,4-dinitrophenylhydrazone (282), C 42.6, H 3.6, N 19.8.

of cold saline. The supernatants, after centrifugation of the mixtures for 30 minutes at 5000 R.P.M. in a refrigerated angle centrifuge, were dialyzed overnight against running tap water and then for 24 hours against several

changes of large volumes of ice-cold distilled water. The centrifugation of the dialyzed solutions resulted in clear supernatants containing approximately 10 mg. of dissolved material per cc. These extracts of rat or mouse liver were almost inactive in the deamination of *dl*-serine in evacuated vessels, unless  $Mg^{++}$  was added.

A similarly prepared extract of rabbit liver in which 1 volume of saline was used for the extraction did not require the activation by magnesium ions; but it is possible that the dialysis was less complete in this case. A selection of data on deamination by such extracts is presented in Table V.

It should be mentioned that quite often completely inactive dialysates (even after addition of  $Mg^{++}$ ) were encountered. The reasons for these

TABLE V  
*Deamination of dl-Serine by Liver Extracts*

1 cc. of liver extract, 1 cc. of 0.1 M phosphate buffer of pH 7.6, and 1 cc. of *dl*-serine solution containing 168  $\gamma$  of serine N were introduced into each vessel, which was subsequently evacuated.  $MgCl_2$ , where added, was present in concentration of 0.005 M. The incubation was for 24 hours at 38°.

Tissue	Substrate	Addition	Ammonia nitrogen	Extent of deamination
			$\gamma$	<i>per cent</i>
Rat liver	<i>dl</i> -Serine	$MgCl_2$	8	0
	"		22	8
	<i>dl</i> -O-Methylserine		110	61
Mouse liver	"	$MgCl_2$	8	0
	<i>dl</i> -Serine		11	0
	"		58	28
Rabbit "	<i>dl</i> -Serine	$MgCl_2$	170	95
	"		9	0
	<i>dl</i> -Serine		138	77

frequent failures are not yet clear. It is probable that the nutritional state of the animals, the duration and manner of the dialysis, and the presence in the dialysates of impurities (*e.g.* glycogen) in varying amounts are responsible. These questions are being studied at present.

*Formation of Pyruvic Acid from Serine by Rat Liver*—13 gm. of rat liver were ground with sand and extracted with 2 volumes of cold saline. The supernatant, after centrifugation of the mixture at 5000 R.P.M. in the cold, was treated with 5 cc. of toluene and incubated at 38° in a nitrogen atmosphere with a solution of 150 mg. of *dl*-serine in 25 cc. of 0.1 M phosphate buffer of pH 7.6 for 16 hours. The filtrate, treated as described before, yielded 30 mg. of *pyruvic acid 2,4-dinitrophenylhydrazone*, which after crystallization from ethyl acetate melted with decomposition at 216°.

$C_6H_{10}O_4N_4$  (268). Calculated, C 40.3, H 3.0; found, C 40.5, H 3.2

The authors are grateful to Dr. H. E. Carter, Dr. V. du Vigneaud, Dr. F. Lipmann, and Dr. H. Waelsch for preparations used in the course of this work, to Dr. T. Rosebury of this College for help and advice with respect to the bacterial preparations, and to Mr. W. Saschek for the microanalyses reported here.

#### SUMMARY

The deamination of serine by *Bacterium coli* is inhibited by replacement of the hydroxylic hydrogen atom. Pyruvic and  $\alpha$ -ketobutyric acids have been identified as end-products of the deamination of serine and threonine respectively by bacteria (*Bacterium coli*, *Pseudomonas pyocyanea*, *Proteus* OX-19, *Clostridium welchii*). The anaerobic deamination of serine by cell-free extracts from mouse, rat, and rabbit livers has been demonstrated to proceed in a similar manner.

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# AN IODOMETRIC DETERMINATION OF METHIONINE\*

By THEODORE F. LAVINE

(From the Lankenau Hospital Research Institute, Philadelphia)

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The numerous problems involving the metabolism of methionine make a simple method for its estimation most important. This investigation is concerned with such a method based on the reversible reaction of iodine with methionine.

Organic sulfides or thio ethers are characterized in their reactions with halogens by the formation of perhalides of the type  $R_2S \cdot X_2$  which are more or less readily hydrolyzed to sulfoxides, according to Equations 1 and 2.



Methionine, by virtue of its thio ether structure, is also capable of these reactions, but it differs from most thio ethers in that the periodide which is formed on reaction with iodine is colorless and water-soluble.<sup>1</sup>

In the case of methionine both of the reactions expressed by Equations 1 and 2 are strongly dependent on the acidity of the solution. With decreasing acidity the rate of formation of the periodide *increases* while its rate of hydrolysis *decreases*. At pH 7, in the presence of a slight excess of iodine, the formation of the periodide takes place in a few minutes, but at pH 9 methionine may be directly titrated with iodine. An increase in the iodide concentration decreases the rate of both reactions, but with a greater practical effect on the hydrolysis of the periodide, since the effect on the formation of the periodide is more pronounced in more acid solutions. Thus, in 1 M KI solution at pH 7 methionine is still converted to the periodide in a few minutes in the presence of a slight excess of  $I_2$ , and at pH 9 the correct end-point is approached slowly on direct titration. However, after a 48 hour interval, in 1 M and 0.05 M KI solutions at pH 7 the amount of sulfoxide formed was 57 and 75 per cent, respectively. Similarly, for a 48 hour period at pH 9 the amount of periodide hydrolyzed to the sulfoxide was 27 and 47 per cent in 1 M and 0.05 M KI, respectively. These figures also

\* Aided by a grant from the McNeil Laboratories, Inc., Philadelphia. A preliminary report of the method was made at the meeting of the American Society of Biological Chemists at Boston, April, 1942. The technical assistance of Jane Homiller is gratefully acknowledged.

<sup>1</sup> The methionine periodide is present in solution as an iodosulfonium hydroxide ( $R-S(I)Me^+OH^-$ ), which will be discussed in a subsequent paper together with its implications as applied to iodine oxidations in general.



illustrate the reduced rate of hydrolysis of the periodide when the acidity of the solution is decreased.

Increasing the iodine concentration increases the rate of formation of the periodide but is without effect on its hydrolysis.<sup>2</sup>

Since the periodide is stable against such reducing agents as thiosulfate or arsenite, any excess iodine is easily removed. Acidification of the resulting colorless solution results in the reversal of the reaction shown by Equation 1 with the liberation of  $I_2$  from the periodide which now may be titrated with thiosulfate.

This behavior with iodine furnishes the basis of a convenient method for the estimation of methionine, since most compounds which consume iodine do so irreversibly with the formation of oxygenated products. Although intermediate oxidation products of sulfur compounds (including methionine sulfoxide) can be reduced by HI with the liberation of  $I_2$ , this is a slow reaction which requires such a high concentration (at least 1 M) of HI that it may be neglected as a factor in this case.

The structural requirement for this reaction (or for any oxidation) is the possession of a pair of free electrons by the reducing atom. Many compounds other than thio ethers ( $R-\ddot{S}-R$ ) possess such a configuration;

e.g., other sulfur compounds ( $R-\ddot{S}-\ddot{S}-R$ , etc.), ethers ( $R-\ddot{O}-R$ ),

aldehydes ( $R-C(H)\ddot{O}:$ ), amines ( $R-\ddot{N}H_2$ ), etc. The most interesting of these is perhaps the amino group, not only because it is possessed by all amino acids, but also because the various classes of amines, especially the quaternary ammonium salts like choline and betaine, are able to form periodides. Also a reaction such as this is involved in the formation of  $NI_3$  from  $NH_3$  and  $I_2$ , and in the formation of the sodium salt of N-chloroglycine (Langheld (8)).

It was found that the use of 1 M KI at pH 7 greatly restricts the formation of periodides by all classes of amines, including the quaternary ammonium compounds, and if periodides are formed they are destroyed by thiosulfate and so present no problem. At pH 7 amino acids exist largely as zwitter ions containing the aminium ion,  $-NH_3^+$ , which does not contain a free pair of electrons, and accordingly amino acids have only slight periodide-forming power at this acidity. However, while this effect is small, it assumes importance in cases in which methionine constitutes only a small

<sup>2</sup> This may not be true in strongly alkaline solutions of low iodide concentration in which hypiodite formation is a factor, since there is some evidence that hypiodite can oxidize methionine periodide to the sulfoxide. This will be treated in detail in a subsequent paper.

fraction of a total of amino acids. In such cases the interference may be evaluated by a blank determination after the methionine has been converted to the sulfoxide which does not form a periodide.<sup>3</sup>

Although *natural* amino acids were not examined extensively, *l*(+)-valine and *l*(+)-glutamic acid from a commercial source were found to be contaminated with methionine (Table III), a further example of the need for caution when absolutely methionine-free conditions are important.

Tryptophane reacts with iodine to form a colored precipitate which must be removed by centrifugation or other means before  $I_2$  can be titrated.

Interference on the part of cysteine, cystine, and many other sulfur-containing compounds is eliminated by the use of 1 M KI. The interference of homocystine can be overcome by converting it to the thiolactone of homocystine. The most serious interference is caused by S-substituted homocystine derivatives and, to a lesser degree, by cystamine and esters of cystine. Fortunately these compounds are rarely encountered except under special experimental conditions.

Methionine sulfoxide, methionine alkylsulfonium ions ( $R_3S^+$ ), N-acylated derivatives of methionine, and deaminated derivatives of methionine (as typified by  $\alpha$ -keto- $\gamma$ -methiobutyric acid) do not form stable periodides at pH 7 in 1 M KI. This indicates that the presence of both the methio and the  $\alpha$ -amino groups is necessary for periodide formation. A somewhat greater specificity is thus obtained than is possible with other existing methods. In the Baernstein (1) procedure of digestion with HI not only will the sulfoxide be reduced to methionine, but the other compounds mentioned should also form methyl iodide to a considerable extent. In the peroxide oxidation of Kolb and Toennies (7) (neither methionine sulfoxide nor sulfonium salts respond) and in the colorimetric procedure of McCarthy and Sullivan (10) (the sulfoxide is negative and the sulfonium salts presumably so), an unaltered methio group seems to be the only requisite, since deaminated methionine is oxidized in the former (Floyd and Medes (5)) and glycylmethionine (an N-acylated derivative) is positive in the latter method.

#### EXPERIMENTAL

*General Procedure*—A 25 to 50 per cent excess of iodine is added to the methionine solution in 1 M KI and buffered at pH 7. The excess iodine is removed after 10 to 20 minutes by  $Na_2S_2O_3$ , the solution is then acidified, and the liberated iodine is titrated with thiosulfate. It is usually advisable to run a blank, especially if other amino acids are present. In this, methionine is oxidized to the sulfoxide (which does not form a periodide) by  $KIO_3$ ,

<sup>3</sup> It is not advisable to conduct the periodide reaction in solutions more acid than pH 6.5 (in which the effect of amino acids would be lessened) since here sulfoxide formation is more rapid.

in 0.5 to 1.0 M HCl solution. After 10 to 20 minutes, the excess  $\text{KIO}_3$  is converted to  $\text{I}_2$  by KI, the solution is neutralized and buffered at pH 7, and the iodine determination carried out as before. The difference between the amounts of iodine liberated in the two cases is a measure of the methionine present.

*Reagents*—These may be varied to accommodate individual needs.

6 M HCl and 2 M HCl.

3 M NaOH.

5 M KI.

The buffer at pH 7 is made by mixing 7 volumes of 1 M  $\text{K}_2\text{HPO}_4$  and 3 volumes of 1 M  $\text{KH}_2\text{PO}_4$ .

Dilute  $\text{NaHSO}_3$  solution (0.1 or 0.2 M).

0.1 N  $\text{KIO}_3$ .

0.1 N  $\text{I}_2$ .

Standardized sodium thiosulfate solution (0.025 N).

The determinations are run in ordinary Erlenmeyer flasks. The reagents are conveniently measured by serological pipettes. Accuracy is important only in the final iodine titration. A dilute (0.01 N)  $\text{I}_2$  solution is convenient for adjusting and correcting end-points.

*Procedure, Blank*—Since the amount of reversible periodide formation by various amino acids other than methionine, which constitutes the blank, is a function of the iodine concentration, it is necessary that this correspond approximately to that in the "determination." Accordingly the "blank" is set up first in order that after the iodate oxidation the iodine concentration may be adjusted to that of the determination. Sufficient 6 N HCl is added to an aliquot (usually 5 ml.) of the unknown so that the final concentration will be from 0.5 to 1 N HCl. There is next added sufficient  $\text{KIO}_3$  so that only a slight yellow color is imparted to the solution (approximately a 50 per cent excess). After 10 to 20 minutes (meanwhile the determination, cf. below, has been set up) the excess iodate (and ICl) is converted to  $\text{I}_2$  by adding 1 ml. of 5 M KI. About one-half of the amount of NaOH necessary for neutralization is then added, followed by a mixture made up to include the balance of the NaOH (if the amount of alkali needed is not excessive, it may all be added with the buffer, etc.), buffer, sufficient 5 M KI to make the final solution 1 M, and sufficient water to make the volume the same as in the determination (cf. below). The usual practice is to add 1 ml. of 1 M buffer (and of course 1 ml. of 5 M KI) for each 5 ml. of the final solution. The above order of addition of the alkali is thought to minimize the danger of a local excess of alkali reacting with iodine to form iodate. If necessary, the iodine concentration is next adjusted to match, by visual comparison, that in the determination by adding dilute  $\text{NaHSO}_3$  dropwise. If the  $\text{I}_2$  in the blank is in great excess, it is better practice to remove most of it before the

neutralization and buffering. After 10 to 20 minutes the solution is titrated as in the determination.

*Determination*—This is set up 5 or 10 minutes after the blank is started so that the  $I_2$  consumption will be largely completed by the time the iodate oxidation in the blank is finished. If the solution under consideration is acid, the amount of alkali needed for neutralization is determined, with methyl red as indicator. After neutralization, if necessary, there is added to an aliquot sufficient buffer and 5 M KI so that there will be 1 ml. of each for every 5 ml. of final solution (including  $I_2$ , etc.). There is next added a 25 to 50 per cent excess of  $I_2$  or enough to impart a definite and persistent  $I_2$  color to the solution.<sup>4</sup> After 10 to 20 minutes the excess iodine is destroyed by adding thiosulfate, with starch as an indicator. The end-point should be exact, although the amount of thiosulfate added is not important except as a measure of the excess  $I_2$  for comparison with that of the blank. There is next added 1 to 1.5 ml. of 2 N HCl for every ml. of buffer used and the liberated  $I_2$  is titrated with thiosulfate. The iodide concentration should be at least 0.5 M at the end of the titration in order to prevent the oxidation of methionine by  $I_2$  in the acidified solution which may amount to a 1 or 2 per cent loss in  $I_2$ . If necessary additional 5 M KI is added after the excess  $I_2$  is removed and *before* the solution is acidified. After the blank is deducted, the methionine content is calculated as follows: ml. of  $Na_2S_2O_3 \times 0.5 \times$  normality of thiosulfate = mm of methionine present. Details of actual determinations are shown in Table IV, and in the section on "Protein hydrolysates."

*Methionine*—The results obtained with solutions of methionine are shown in Table I. With a 20 per cent excess of  $I_2$  the methionine periodide formation is complete within 10 minutes, while 20 minutes are required if a lesser amount of  $I_2$  is present. In case of a very low excess of  $I_2$  (10 per cent) the theoretical amount of periodide may not be attained because of its hydrolysis to the sulfoxide, which amounts to approximately 1 per cent of the methionine in an hour's time under the conditions described. The precision of the method is practically that of the starch-iodine-thiosulfate titration under similar conditions of acidity and iodide concentration. Assuming this to be  $\pm 0.01$  ml. of 0.025 N  $Na_2S_2O_3$  (corresponding to  $\pm 1.25 \times 10^{-4}$  mm or  $\pm 0.019$  mg. of methionine), the accuracy of the various determinations of Table I was found in most cases to be within the limits of precision for the 10 or 20 minute interval with aliquots containing amounts of methionine ranging from 1.5 mg. (precision  $\pm 1$  per cent) to 14.9 mg. (precision  $\pm 0.1$  per cent). The purity of the methionine was established by

<sup>4</sup> Occasionally it is necessary to add more  $I_2$  after a few minutes. An approximation of the amount of  $I_2$  needed may be obtained by direct titration with iodine of an aliquot buffered at pH 9.

H<sub>2</sub>O<sub>2</sub> oxidation (Kolb and Toennies (7)) and by perchloric acid titration (Toennies and Callan (13)). With an individual sample of 22.5 mg. of

TABLE I  
*Formation and Stability of Methionine Periodide in 1 M KI Buffered at pH 7*

Methionine	I <sub>2</sub>	Periodide I <sub>2</sub> *					Calculated precision
		5 min	10 min	20 min.	40 min	60 min	
<i>moles per l.</i>	<i>moles per l.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.002	0.0024		99	101	101	101	±1
	0.0040		101	101	101	98	±1
	0.0080		100	101	100	99	±1
0.005	0.0053		97.9	99.5	99.5	99.1	±0.25
	0.006	97.5	99.5	99.8	99.5	98.7	±0.5
	0.0075	99.9	100.3	99.9	99.9	99.1	±0.2
	0.010	100.3	100.3	99.9	99.5	99.1	±0.5
0.010	0.0106		99.7	99.7	99.1	98.5	±0.25
	0.0120		100.0		99.3		±0.25
0.020	0.021	98.7	98.8	98.7			±0.10
	0.025	99.9	99.9	99.2			±0.10

\* This is the reversibly bound I<sub>2</sub> which is released on acidification of the solution after the excess I<sub>2</sub> has been destroyed. The results are expressed as percentages of the theoretical amount of methionine.

TABLE II  
*Reversible Periodide Formation by Glycine in 1 M KI*

The volume in each case was 6.5 ml. Increasing the volume to 25 ml. results in a slight decrease in periodide I<sub>2</sub> (0.01 ml.). The periodide I<sub>2</sub> was released on acidification of the solutions after the excess I<sub>2</sub> had been destroyed.

The results are expressed in terms of ml. of 0.025 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

Glycine	pH	Excess I <sub>2</sub>	Periodide I <sub>2</sub>
<i>mM</i>			
1	7	0.21	0.00
		0.40	0.01
		0.77	0.04
		1.61	0.10
1	7	0.77	0.04
2		0.81	0.07
4		0.71	0.09
1	9	0.12	0.09
		0.30	0.17
		0.56	0.33
		1.16	0.47

methionine (made 0.003 M in methionine and 0.0045 M in I<sub>2</sub>) a value 99.8 per cent of the theoretical was obtained.

*Amino Acids*—The ability of amino acids to form periodides is exemplified by the behavior of glycine as shown in Table II. The necessity for

adjustment of the excess  $I_2$  of the blank to that in the determination is based on the increase in periodide  $I_2$  with increasing  $I_2$  concentration.

In order to evaluate the interference by the various amino acids as shown in Table III, individual samples equivalent to 1 mm were taken and  $I_2$

TABLE III  
*Periodide Formation by Amino Acids*

The periodide  $I_2$  figures represent ml. of 0.024 N  $Na_2S_2O_3$  required by the  $I_2$  liberated in acid solution after the excess  $I_2$  has been discharged. 1 mm of methionine is equivalent to 83.3 ml. Galactose, which is also a constituent of casein, does not form a periodide. The prefix *l*- denotes the natural and *d*- the unnatural enantiomorph of an amino acid. The *l*(+)-valine and *d*-valine were Hoffmann-La Roche products. The *l*(+)-glutamic acid was a Corn Products preparation recrystallized from water and possessed an equivalent weight of 146.8 by perchloric acid titration. The figures in the third and sixth columns under "Amino acid mixture" show the amount of each amino acid added to a mixture which was dissolved in 200 ml. of solution containing 28 ml. of 2 N HCl. The acidity of the final solution was 0.40 M (with methyl red as indicator).

Amino acid	Periodide $I_2$ (0.012 M)	Amino acid mixture	Amino acid	Periodide $I_2$ (0.012 M)	Amino acid mixture
1 mm of amino acid in 20 ml of 1 M KI, pH 7, was let stand for 20 min. with 0.1 ml. of 0.1 N $I_2$					
$NH_4Cl$	0	630	<i>dl</i> -Serine	0.035	450
<i>dl</i> -Alanine	0	250	<i>l</i> (+)-Arginine·HCl	0.05	570
<i>l</i> (-)-Tyrosine (ppt.)	0	600	<i>l</i> (+)-Lysine·2HCl	0.05	1030
<i>l</i> (-)-Aspartic acid	0.01	370	<i>dl</i> -Histidine·HCl	0.06	300
<i>l</i> (-)-Proline	0.015	820	<i>dl</i> -Threonine	0.06	340
<i>d</i> -Valine	0.02	720	<i>l</i> (-)-Hydroxyproline	0.07	200
Glycine	0.02	330	$\frac{2}{3}$ <i>l</i> -Cystine	0.10	40
<i>dl</i> -Phenylalanine	0.02	450	<i>l</i> (+)-Glutamic acid	Cf. below	1980
<i>dl</i> -Isoleucine	0.03	150	<i>d</i> -Leucine	0.13	720

1 mm of amino acid treated as above with 0.5 ml. of 0.1 N  $I_2$

				Methionine per cent
<i>l</i> (+)-Valine	0.29	After iodate treatment		0.28
<i>d</i> -Valine	0.06			
<i>d</i> -Leucine	0.11	" "	0.12	0
<i>l</i> (+)-Glutamic acid	0.13	" "	0.05	0.1

added to a slight excess. After 20 minutes the excess  $I_2$  was destroyed and the amount of periodide  $I_2$  measured as usual. When possible, the inactive or unnatural modification was used in order to avoid the possibility of contamination by methionine. The necessity for this is apparent from the cases of *l*(+)-valine and *l*(+)-glutamic acid in which the difference between

the periodide content before and after iodate treatment showed methionine to be present, which was confirmed by a qualitative test for sulfur. The relatively high periodide  $I_2$  value obtained with *d*-leucine was unchanged by iodate treatment and therefore was not due to methionine.

The amino acids were then combined in amounts shown in the third and sixth columns of Table III, to approximate a hydrolysate containing 5 gm.

TABLE IV

*Estimation of Methionine in Amino Acid Mixtures*

The results are expressed in terms of ml. of 0.024 N  $Na_2S_2O_3$ .

Solution No.	Treatment*	Excess $I_2$	Periodide $I_2$
1	1 ml. 0.1 N $KIO_3$	1.83	0.14
2	2 " 0.1 " " $I_2$ adjusted with $NaHSO_3$	2.13	0.15
3	1 " 0.05 M methionine; 3 ml. 0.1 N $KIO_3$ ; $I_2$ adjusted with $NaHSO_3$	1.89	0.16
4	0.5 ml. 0.1 N $I_2$	1.90	0.15
5	1 ml. 0.0500 M methionine; 1.5 ml. 0.1 N $I_2$	1.96	4.33

\* Each solution contained 5 ml. of the "Amino acid mixture" (cf. Table III). Solutions 1, 2, and 3 were set up 10 to 15 minutes before Solution 4.

*Solution 1*—1 ml. of 6 N HCl and the amount of  $KIO_3$  shown were added to the 5 ml. of "Amino acid mixture" and after 20 minutes 1 ml. of 5 M KI followed by a mixture composed of 4.23 ml. of 1.88 M NaOH, 5 ml. of buffer, pH 7, 4 ml. of 5 M KI, and 4 ml. of  $H_2O$  (final total volume 25 ml.). After the solution had stood for 20 minutes, the excess  $I_2$  was removed with 0.02400 N  $Na_2S_2O_3$  and after the addition of 5 ml. of 2 N HCl the periodide  $I_2$  was titrated with  $Na_2S_2O_3$ .

*Solution 2*—This was treated the same as Solution 1 except that 1.2 ml. of 6 M HCl, 4.87 ml. of NaOH, and 3.4 ml. of  $H_2O$  were used in their respective places. The  $I_2$  concentration was brought to that of Solutions 1 and 4 by adding 0.2 M  $NaHSO_3$  immediately after adjustment to pH 7.

*Solution 3*—This was prepared and treated like Solution 2 except that 1.6 ml. of 6 M HCl, 6.1 ml. of NaOH, and no  $H_2O$  were used.

*Solution 4*—In addition to 5 ml. of "Amino acid mixture" and 0.5 ml. of  $I_2$  this contained 5 ml. of buffer, 5 ml. of 5 M KI, 1.03 ml. of 1.88 M NaOH, and 9 ml. of  $H_2O$ . The excess  $I_2$  and periodide  $I_2$  were estimated after 20 minutes.

*Solution 5*—This was treated the same as Solution 4 except that 7 instead of 9 ml. of  $H_2O$  were used. Deduction of the blank (0.15 ml.) from the determination (4.33 ml.) yields a figure (4.18 ml.) corresponding to 100.5 per cent of the theoretical amount of methionine added.

of casein per 100 ml. Periodide formation before and after iodate treatment was measured on 5 ml. aliquots of this solution with the results reported in Table IV.

The validity of the iodate blanks (Solutions 1, 2, and 3) is established by their agreement with the periodide determination in Solution 4. The estimation of 0.05 mm of methionine added to this synthetic mixture (Solution 5) resulted in a value 100.5 per cent of the theoretical. The periodide

value of the mixture was constant over the interval from 10 to 40 minutes, although the  $I_2$  consumption continued to increase.

**Tryptophane**—Tryptophane, if present, forms a brown precipitate with  $I_2$  at pH 7, which obscures the end-point. This in itself is not a serious difficulty, since the precipitate is easily removed by centrifugation and an aliquot of the supernatant may be taken for titration. However, it was found that in the blank the products of oxidation of tryptophane by iodate (somewhat more than 2 atoms of oxygen per mole of tryptophane are consumed) consume iodine at pH 7 with the formation of a slight brown color with a slightly higher periodide formation than is found in a solution treated originally with  $I_2$  at pH 7 and centrifuged. This causes a negative error in the determination of methionine.<sup>5</sup> Thus, a mixture containing 5 ml. of the "Amino acid mixture," 0.1 mm of tryptophane, and 0.05 mm of methionine was analyzed as outlined and found to contain 0.0465 mm of methionine or 93 per cent of theoretical value.

**Protein Hydrolysates**—On the basis of the results with amino acids there would appear to be no interfering substances in protein hydrolysates which are not evaluated by the blank. This is verified by the results with actual protein hydrolysates. One such protein will be treated in detail to elucidate the details of the method. The results on this and other proteins are summarized in Table V.

**Casein**—A sample weighing 1.943 gm. (dry weight) was hydrolyzed 7 hours with 20 ml. of 6 N HCl.<sup>6</sup> After being concentrated *in vacuo* two times, it was decolorized with 0.2 gm. of carboraffin and diluted to 50 ml. (0.41 N in HCl). To two 5 ml. aliquots were added 1 ml. of 2 N NaOH, 5 ml. of buffer at pH 7, 4 ml. of 5 M KI, 4 ml. of  $H_2O$ , and 1.0 and 1.2 ml. of 0.1 N  $I_2$  respectively.<sup>7</sup> After 20 minutes the excess  $I_2$  amounted to 0.47 and 1.25

<sup>5</sup> Mr. J. J. Kolb of this Institute for an exact determination corrected for this by a separate tryptophane estimation and measurement of the effect of this amount of tryptophane

<sup>6</sup> A rapid and efficient method of hydrolysis consists of hydrolysis in 6 N HCl with a ratio of HCl to N of 2:1. Practically this amounts to the addition of 1.83 ml. of 12 N HCl and 1 ml. of  $H_2O$  to 1 gm. of protein. The resulting mass is heated on a water bath until it becomes liquid with the bulk of the protein in solution. The flask should not be swirled, or the protein will adhere to the walls of the flask and become resistant to solution especially if above the liquid level. The mixture is then heated on an oil or sand bath for 4 hours (boiling temperature for casein is 109°). Heating for 8 hours results in only a slight increase (2 per cent) in amino N or methionine for such diverse proteins as egg albumin (Toennies, unpublished data), whole rats (Floyd and Medes, unpublished data), and casein. Theoretically the activity of HCl, the rate of hydrolysis, and the boiling point are increased with increasing concentration of amino acids, etc. A point of analytical advantage results from the fact that the final solution contains an excess of only 1 HCl per N.

<sup>7</sup> It is customary to run duplicate determinations with varying amounts of excess  $I_2$ , which by their agreement will confirm the absence of errors due to cystine, etc.; cf. the section on "Periodide formation by various compounds."



TABLE V  
Methionine Content of Proteins

Sample No.*	Protein	Methionine				
		Periodide	Homocystine		Volatile iodide	
			Baernstein (1, 2)	Kassell and Brand (6)†	Baernstein (1, 2)	Kassell and Brand (6)‡
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Egg albumin	4.58	4.49	4.55	5.07	4.89
2	"	3.76		2.74		3.44
3	Lactalbumin	2.41	2.32§	2.58	2.45§	2.63
3A	"	2.40				
4	"	2.35				
4A	"	2.36				
4B	"	2.39				
5	Casein	2.86	3.10§	2.73	3.31§	2.95
6	"	2.85				
6A	"	2.85				
6B	"	2.89				
6C	"	2.89				
7	Zein	1.68	2.46§		2.58§	

\* Sample 1—Egg albumin C, Kassell and Brand (6), kindly furnished by Dr. Brand. 0.675 gm. was hydrolyzed 19 hours with 15 ml. of 6 N HCl, decolorized with 0.15 gm. of carboraffin after concentration *in vacuo*, and diluted to 50 ml.; 5 ml. aliquots were used for analysis.

Sample 2—Merck's powdered egg albumin (14.1 per cent N). 10 gm. were hydrolyzed for 21.5 hours with 14.4 ml. of 12 N HCl and 13 ml. of H<sub>2</sub>O, decolorized after concentration *in vacuo*, and diluted to 200 ml.; 3 ml. aliquots were used for analysis. The homocystine and volatile iodide determinations were carried out by Mr. T. P. Callan of this Institute.

Sample 3—Labco 7-HAAX, kindly supplied by Dr. Brand. 1.89 gm. (dry) were hydrolyzed for 8 hours in 10 ml. of 6 N HCl, twice concentrated *in vacuo*, twice decolorized with 0.3 gm. of carboraffin, and diluted to 100 ml.; 10 ml. aliquots were used for analysis.

Sample 3A—0.945 gm. of the same protein was hydrolyzed for 20 hours with 8 ml. of 6 N HCl, twice concentrated *in vacuo*, decolorized with 0.3 gm. of carboraffin, and diluted to 50 ml.; 5 ml. aliquots were used for analysis.

Sample 4—Labco 7-HAAX kindly furnished by Dr. G. C. Supplee. 18.38 gm. (dry) with 18.4 ml. of H<sub>2</sub>O and 33.7 ml. of 12 N HCl were heated for 7 hours on a water bath, hydrolyzed for 7 hours, and diluted to 200 ml. 50 ml. were decolorized twice with 1 gm. of carboraffin, diluted to 100 ml., and 5 ml. aliquots taken for analysis.

Sample 4A—A second 50 ml. aliquot of Sample 4 plus 5 ml. of 0.0492 M methionine was decolorized as Number 4 was. Deduction of the calculated amount of methionine leaves the value reported in Table V and shows that no methionine was lost on decolorization.

Sample 4B—A third 50 ml. aliquot of Sample 4 was heated for 1 hour with 1.18 gm. of Sn dust, filtered, concentrated over a free flame, the tin precipitated with H<sub>2</sub>S, the H<sub>2</sub>S expelled by CO<sub>2</sub>, and the resulting solution diluted to 100 ml.; 5 ml. aliquots were used for analysis. The cysteine content as determined by I<sub>2</sub> oxidation in 1 M

TABLE V—*Concluded*

HI (Lavine (9)) was 3.09 per cent, in agreement with the figure (3.09 per cent) obtained by Kassell and Brand (6).

*Sample 5*—Labco vitamin-free casein kindly supplied by Dr. Brand (cf. the text for details of analysis).

*Sample 6*—Labco vitamin-free casein kindly supplied by Dr. G. C. Supplee. 19.34 gm. of dry casein were heated on a water bath for 5 hours with 35.5 ml. of 12 N HCl and 19.3 ml. of H<sub>2</sub>O and then hydrolyzed for 4 hours on an oil bath. (Some fatty matter appeared in the condenser.) An aliquot by weight corresponding to 10.06 gm. of casein was diluted to 100 ml. (2.1 N in HCl) Methionine was estimated directly on 2 ml. aliquots, appropriately diluted and neutralized aliquots being used for reference color standards.

*Sample 6A*—A 40 ml. aliquot of Sample 6 was decolorized by 1 gm. of carboraffin and diluted to 100 ml.; 5 ml. aliquots were used for analysis.

*Sample 6B*—The remainder of the undiluted solution of Sample 6 (9.215 gm. of casein) was hydrolyzed an additional 4 hours and diluted to 100 ml. A 40 ml. aliquot was decolorized with 1 gm. of carboraffin and 5 ml. aliquots were used for analysis.

*Sample 6C*—Another 40 ml. aliquot (0.8 N in HCl) was heated with 1.18 gm. of tin dust, filtered, the tin precipitated by H<sub>2</sub>S, filtered, the H<sub>2</sub>S expelled by CO<sub>2</sub>, and the solution diluted to 100 ml. 5 ml. aliquots were used for analysis.

*Sample 7*—Cf. the text.

† These are *uncorrected* figures, which must be multiplied by 1.12 to attain correct values, according to Kassell and Brand (6).

‡ These are *uncorrected* values, the correction factor is 1.067 according to Kassell and Brand (6).

§ Harris product.

ml. of 0.02390 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> for the two aliquots and the respective values for periodide I<sub>2</sub> were 3.28 and 3.30 ml. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. For the blanks, which were set up 10 minutes before the determinations, there were added to two 5 ml. aliquots 1.2 ml. of 6 N HCl and 1.6 and 1.8 ml. of 0.1 N KIO<sub>3</sub>. After 20 minutes 2 ml. of 2 N NaOH and 2 ml. of 5 M KI were added to each aliquot followed in each case by a mixture containing 2 ml. of 2 N NaOH, 2 ml. of 5 M KI, and 4 ml. of buffer (pH 7). Since the I<sub>2</sub> content of these solutions approximately matched that of the above determinations, no adjustment with NaHSO<sub>3</sub> was necessary. After 20 minutes the values for excess I<sub>2</sub> amounted to 0.84 and 1.22 ml. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, and for the periodide I<sub>2</sub>, 0.17 and 0.18 ml. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Deduction of these latter values from the appropriate determinations yields 3.11 and 3.12 ml. of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, corresponding to a methionine content amounting to 2.85 and 2.86 per cent of the dry casein.

In some cases (casein and zein) the methionine determination was carried out on the untreated hydrolysate. This is feasible only if the solution is light enough in color so that a meniscus may be easily read. Even so it is necessary that reference standards be prepared from the hydrolysate for the end-point at pH 7 and in the acid solution. While this is admittedly a difficult titration, it was performed with satisfactory results in the two cases mentioned to determine whether decolorization by charcoal involved any

loss in methionine. Since no evidence for such loss was indicated,<sup>8</sup> it is much more convenient to decolorize the sample.

*Zein*—(This was kindly furnished by the Corn Products Refining Company.) 16.42 gm. of dry protein (5.98 per cent water) were heated 6½ hours on a water bath with 30 ml. of 12 N HCl and 16.4 ml. of H<sub>2</sub>O. After being heated 7 hours on an oil bath the solution was filtered and diluted to 200 ml. Analyses carried out with 1 ml. aliquots indicated 15.99 per cent nitrogen. Methionine estimations carried out on the untreated hydrolysate showed 1.61 per cent methionine, on a fraction decolorized by carboraffin 1.68 per cent, and on a fraction decolorized by tin and H<sub>2</sub>S 1.63 per cent methionine and 0.83 per cent cystine. The methionine figure is considerably lower than that obtained by Baernstein (2). The cystine figure is also lower than the 1.05 per cent obtained by Baernstein, but is in fair agreement with figures obtained by Vickery and White (14); *i.e.* 0.91 per cent by cuprous precipitate, 0.85 per cent by the Sullivan method.

*Oxidized Casein (Toennies (12))*—This modified casein is characterized by the oxidation of methionine and tryptophane and is of interest because of its high blank value, amounting to 1 ml. of 0.024 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> per 0.25 gm. of protein which is unchanged by iodate oxidation. The blank is attributed to the oxidation products of tryptophane, since neither the sulfoxide nor the sulfone of methionine forms a periodide to any appreciable extent. However, the blank adequately compensates for this unknown periodide and no methionine was indicated within the limits of error of titration.

*Enzyme Digests*—The method has also been applied to trichloroacetic acid filtrates from protein-enzyme digests with satisfactory results (Floyd and Medes (5)). With these solutions the blank is made 0.5 to 1 N in HCl as usual, and sufficient alkali is added to neutralize both the HCl and the trichloroacetic acid when adjustment to pH 7 is made.

#### DISCUSSION

The primary purpose of this investigation was the estimation of free or preformed methionine present in solution and not the determination of the methionine content of proteins. The latter is an entirely separate problem which involves consideration of losses inherent in hydrolysis and manipulation before the final solution is obtained for analysis.

Although it is thought that the use of correction factors of the order of 10 per cent requires a method of considerable precision for accuracy of final results, there does exist considerable evidence that some methionine sulfur is lost by HI hydrolysis (Kassell and Brand (6); Beach and Teague (4)). Accordingly, the periodide values for the methionine content of proteins

<sup>8</sup> Mr. N. F. Floyd of this Institute by independent experiments has also demonstrated that treatment of methionine in 0.4 N HCl with charcoal involved no loss.

would seem to be low, since in general they are only slightly higher than the uncorrected homocysteine values. At the same time it is felt that the periodide method permits estimation of all the methionine present in the HCl hydrolysates. The cause of this discrepancy may reside in the treatment of the protein, although it seems unlikely, since Bailey (3) found that the Baernstein procedure (volatile iodide) yielded identical results when applied either directly to a protein or to an HCl hydrolysate. Bailey also found that humin removal occasioned no loss in methionine. In addition it has been shown in the present paper that decolorization by charcoal in acid solution entails no loss in methionine.<sup>8</sup>

There remains the possibility that in the HCl hydrolysate, some of the methionine is present in a modified form (as a sulfonium salt, etc.) that does not form a periodide but is destroyed by HI. In the case of zein divergent values of a completely different order of magnitude were obtained, indicating either that this last factor is here operating very strongly or that some other sulfur compound is present that behaves like methionine in the Baernstein procedure.

*Periodide Formation by Various Compounds*—The following compounds<sup>9</sup> did not form periodides in any appreciable amounts under the conditions employed in the methionine determination; *i.e.*, in 1 M KI buffered at pH 7. The compounds marked with an asterisk when present with a large excess of I<sub>2</sub> (5 or 10 moles of I<sub>2</sub> per mole of compound) form periodides to a slight extent (0.1 to 0.2 mole of periodide I<sub>2</sub> per mole of compound): methionine sulfoxide; methionine sulfone; methionine methylsulfonium iodide\*; N-formyl-, acetyl-, or benzoylmethionine; N-formylmethionine methylsulfonium bromide; S-benzyl-N-dibenzylhomocysteine (this compound forms an insoluble periodide which is destroyed on addition of thiosulfate); homocysteine-cysteine-thio ether\*; S-benzylpentacysteine\*; S-methyl-, ethyl-, or benzylcysteine; S-benzylglutathione; carboxymethylcysteine; lanthionine; 2,4-dinitrophenylhydrazone of  $\gamma$ -methio- $\alpha$ -ketobutyric acid; barium  $\gamma$ -methio- $\alpha$ -ketobutyrate;  $\gamma$ -methiobutyric acid; dialanycystine\*; diformylcystine;  $\alpha$ -hydroxy- $\beta$ -dithiobarium dipropionate; di- $\alpha$ -bromopropionylcystine\*; *p*-bromophenylmercapturic acid; N-formyl-S-*p*-bromobenzyl-*dl*-cysteine; creatine; creatinine; choline.

The list reveals that on the basis of its periodide-forming powers, methionine may be distinguished from its sulfoxide and sulfone, from its sulfonium salts, from its acylated derivatives, from its deaminated derivatives (*cf.* keto derivatives and  $\gamma$ -methiobutyric acid), and from its homologues, as

<sup>9</sup> The author is indebted to the following for kindly supplying samples of the various compounds listed above and in Table VI: Dr. E. Brand, Dr. V. du Vigneaud, Mr. N. F. Floyd, Dr. D. B. Jones, Dr. G. Medes, Mr. W. Sakami, Dr. J. A. Stekol, Dr. G. Toennies, Dr. H. Waelsch.

exemplified by S-benzylcysteine and S-benzylpentacysteine. Such N compounds as choline, creatine, and creatinine were without effect under the conditions described.

In Table VI are listed the results obtained on various other compounds.<sup>10</sup> The amount of oxidation and periodide formation were followed as a function of time, but only the results for a 15 minute interval are reported. The oxidation of homocystine, cysteine, cystine, and even cysteic acid by I<sub>2</sub>

TABLE VI

*I<sub>2</sub> Consumption and Periodide Formation per Mole of Various Compounds at pH 7*  
The measurements were made after a 15 minute interval.

Compound	Molarity	0.02 M I <sub>2</sub>				0.01 M I <sub>2</sub>		0.004 M I <sub>2</sub>	
		0.08 M KI		1 M KI		1 M KI		1 M KI	
		Total I <sub>2</sub> consumed	Periodide I <sub>2</sub>	Total I <sub>2</sub> consumed	Periodide I <sub>2</sub>	Total I <sub>2</sub> consumed	Periodide I <sub>2</sub>	Total I <sub>2</sub> consumed	Periodide I <sub>2</sub>
		moles	moles	moles	moles	moles	moles	moles	moles
Cysteine	0.004	1.66	0.19	1.43	0.12	1.02	0.065	0.78	0.045
Cystine	0.002	1.68	0.44	1.27	0.19	0.57	0.09	0.30	0.05
Cysteinesulfinic acid	0.01	1.09	0.06	1.00	0.03	0.84	0.05		
Cystine disulfoxide	0.003	3.64	0.60	3.69	0.58	2.98	0.54		
Diethyl ester of <i>l</i> -cystine*	0.002	3.81	0.31	1.36	0.58	0.85	0.48	0.23	0.19
Cystamine*	0.002	3.23	0.91	0.81	0.74	0.51	0.49	0.13	0.13
“ disulfoxide	0.003	2.11	1.06	1.93	1.16				
S-Benzyl- <i>dl</i> -homocysteine	0.004	0.97	0.97	0.97	0.97				
Homocysteine	0.002	2.16	0.61	1.66	0.37				
Homocystine	0.002	3.58	1.74	3.17	1.25	1.15			
Homocysteine thiolactone†	0.004	1.21	0.18	0.49	0.32				
δ-Methiobutylamine*	0.004	1.00	0.84	0.90	0.71			0.90	0.69

\* Forms a precipitate with I<sub>2</sub> in 0.08 M KI, which is destroyed by Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.

† When the ratio of I<sub>2</sub> to thiolactone is reduced to 0.25:1, only 0.037 mole of periodide I<sub>2</sub> is formed per mole of thiolactone. The oxidation of the thiolactone (0.004 M) by iodate (0.02 N) in 1 N HCl is slow, 0.06, 0.24, 0.47 atom of O being consumed per mole in 5, 27, and 60 minutes, respectively.

at pH 7 leads to extensive decomposition with the formation of sulfate and iodoform.

The ability of cystine and cysteine to form periodides decreases with decreasing I<sub>2</sub> concentration. On the basis of the sulfur content, cystine is less effective in forming periodides than is cysteine. For this reason when cysteine is present it is thought advisable first to oxidize it to cystine by adding a slight excess of I<sub>2</sub> to the acid solution, which contains all of the KI to be added for the subsequent methionine determination.

<sup>10</sup> These as well as other compounds will be discussed more fully in a succeeding paper elucidating structural effects on the reactivity of sulfur.

Cystine disulfoxide as such would interfere seriously, but practically does not because of its limited stability and ready decomposition into the sulfinic acid and cystine at pH 7.

Compounds which might interfere include alkyl esters of cystine (and presumably its homologues) and cystamine and its disulfoxide. However, their ability to form periodides decreases sharply with decreasing  $I_2$  concentration, in contrast with methionine, so that even in the presence of these compounds results of comparative value should be possible. The increased ability of these compounds (in which the effect of the carboxyl group is lessened) to form periodides is indicated by their precipitation in solutions of low iodide content. The behavior of  $\delta$ -methiobutylamine suggests that decarboxylated methionine ( $\gamma$ -methiopropylamine) will react like methionine.

S-Alkyl- or arylhomocysteine derivatives behave as methionine does, as far as periodide formation is concerned, and should be absent.

Homocysteine also interferes but its effect may be circumvented by reduction to homocysteine followed by conversion to the thiolactone. The thiolactone forms a periodide to a very limited extent which, however, may be evaluated by the blank, since the thiolactone is but slowly oxidized by iodate. Because methionine-homocysteine interrelationships are of considerable interest at the present time, the following method is described for the estimation of each of these substances.

*Estimation of Homocysteine and Methionine*—The method is based on the reduction of homocystine to homocysteine by zinc and estimation of homocysteine by  $I_2$  oxidation in 1 M HI (Lavine (9)). Any cystine present will also be reduced by this treatment and estimated. The homocysteine is then converted to the thiolactone by heating in concentrated HCl (Riegel and du Vigneaud (11)), after which only cysteine is estimated by the  $I_2$  oxidation in 1 M HI. The methionine determination and blank are carried out as previously described to measure the methionine present.

An aliquot is made 0.6 N in HCl and an equivalent amount of zinc dust added. After 45 minutes the mixture is filtered if necessary and diluted to volume and an aliquot taken for the estimation of homocysteine. Another aliquot is placed in a small distillation flask together with 3 ml. of concentrated HCl and the whole evaporated *in vacuo* to 3 to 5 ml.<sup>11</sup> The vacuum is then released and the solution heated at 80–90° for 15 minutes, after which the distillation *in vacuo* is resumed to semidryness. Near the end of the

<sup>11</sup> A convenient capillary which this author has not seen described, but which has been in use in this laboratory for some time, consists of a section of glass tubing of 4 mm. bore which passes through the rubber stopper to the bottom of the distilling flask. To the top of this is attached by rubber tubing a small piece of thermometer tubing. This device will not clog or break, is easily washed, and may be raised or lowered. A small funnel may be attached for adding octyl alcohol.

distillation it is advisable to remove the water bath. The flask and the tube are rinsed out and diluted to volume. If desired, cysteine may be measured at this point by the  $I_2$  oxidation in 1 M HI. The methionine is measured as usual; the blank is necessary. Although zinc phosphate is precipitated at pH 7, it does not interfere with the titration. It is, however, advisable to use 2 instead of 1 ml. of buffer per 5 ml. of solution.

The various phases of the above procedure were verified experimentally as follows:

*Thiolactone*—For 4 ml. of 0.02 M thiolactone the iodate blank amounted to 0.39 ml. of 0.0244 N  $Na_2S_2O_3$  (excess  $I_2$ , 3.32 ml.) and the methionine procedure showed 0.33 ml. of  $Na_2S_2O_3$  (excess  $I_2$ , 3.53 ml.). Thus the periodide formation by the thiolactone is essentially the same before and after iodate treatment. The corresponding determinations carried out with the addition of 1 ml. of 0.0500 M methionine consumed 4.30 ml. of  $Na_2S_2O_3$  for the periodide  $I_2$  (excess  $I_2$ , 3.60 ml.) with the blank amounting to 0.32 ml. (excess  $I_2$ , 3.52 ml.). The difference, or 3.98 ml. of 0.02442 N  $Na_2S_2O_3$  indicates 0.0486 mm of methionine, or 97.2 per cent of the theoretical value.

*Reduction of Homocystine*—0.1341 gm. (0.5 mm) of homocystine was washed into an Erlenmeyer flask with 5 ml. of 2 N HCl and 9 ml. of  $H_2O$  and 0.33 gm. (5 mm) of zinc dust added. After 40 minutes the solution was filtered and diluted to 50 ml. When 3 ml. aliquots were used, the solution was found to be 0.0135 N in HCl and 0.02018 M in homocystine or 100.9 per cent of the theoretical.<sup>12</sup>

*Thiolactone Formation*—10 ml. of the above solution together with 2 ml. of 0.0500 M methionine were transferred to a 50 ml. distillation flask. After the addition of 4 ml. of 10 N HCl, the solution was concentrated *in vacuo* to 3 to 5 ml. (this required 17 minutes), heated at 80–85° for 15 minutes, and then evaporated *in vacuo* to semidryness. After dilution to 25 ml. aliquots of 10 ml. were taken for the  $I_2$ -1 M HI oxidation and the methionine determination. No homocystine was present and 98.0 per cent of the theoretical amount of methionine was indicated after deduction of the blank (from a separate determination).

In another case, 5 ml. of 0.01 M homocystine, 2 ml. of 0.0500 M methionine, and 3 ml. of 2 N HCl were placed in a 50 ml. distillation flask and reduction carried out with 0.20 gm. of zinc dust. After 15 minutes 1 ml. of 10 N HCl was added and the reduction allowed to proceed for an additional 15 minutes. Then 3 ml. of 10 N HCl were added and the solution concen-

<sup>12</sup> After the solution stood 24 hours at room temperature a decrease of only 4.2 per cent of the homocystine was observed, which indicates that this substance is not as labile as commonly thought, although the presence of zinc chloride may be a stabilizing factor. There was no loss of homocystine when the solution was simply evaporated to semidryness *in vacuo*.

trated *in vacuo* on the water bath. The unused zinc disappeared during concentration. On reaching a volume of from 3 to 5 ml. the solution was heated to 75° for 20 minutes and then evaporated to semidryness. After dilution to 25 ml. the solution was found to be 0.0385 M in acid (with methyl red as indicator) and to contain 101.3 per cent of the theoretical amount of methionine (with a 5 ml. aliquot). (The periodide  $I_2$  of the blank amounted to 0.06 ml. of 0.02442 N  $Na_2S_2O_3$ ; excess  $I_2$ , 2.08 ml. The periodide  $I_2$  in the determination amounted to 1.72 ml. of  $Na_2S_2O_3$ ; excess  $I_2$ , 2.31 ml.)

#### SUMMARY

A rapid and convenient method has been developed for the estimation of methionine based on its reversible reaction with iodine.

By carrying out the reaction at pH 7 in 1 M KI one achieves considerable specificity with the method, inasmuch as such closely related compounds as the sulfoxide, sulfonium salts, and acylated and deaminated derivatives of methionine do not undergo the reaction.

The interference, or periodide-forming powers, of amino acids is evaluated by a "blank" after the methionine has been oxidized to the sulfoxide by iodate in 1 M HCl. The difference in the amount of reversibly bound  $I_2$  before and after the iodate treatment provides a measure of the methionine.

The presence of tryptophane or homocystine requires modifications in the procedure which are described.

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# THE ESTIMATION OF ATABRINE IN BIOLOGICAL FLUIDS AND TISSUES\*

By BERNARD B. BRODIE AND SIDNEY UDENFRIEND

(From the Research Service, Third (New York University) Medical Division, Goldwater Memorial Hospital, New York, and the Department of Medicine, New York University College of Medicine, New York)

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The following are two methods for the estimation of atabrine in biological fluids and tissues through the measurement of its fluorescence in an acidic environment. The methods have a high degree of specificity in that they exclude from the estimation the many fluorescent degradation products of atabrine as well as the naturally occurring fluorescent components of biological fluids and tissues.

The first method is a double extraction procedure. It is wholly satisfactory for the precise estimation of the concentration of atabrine in the plasma, whole blood, tissue, and urine of patients on the usual régimes of suppressive or definitive atabrine therapy. The procedure gives recoveries of added atabrine which average 98 per cent with amounts as low as 0.1  $\gamma$ . Variation at this level is usually less than 5 per cent, and is minimized when larger amounts of atabrine are present. The precision of the estimation decreases when smaller quantities are present.

The second procedure involves only a single extraction. Its speed and simplicity recommend it for use when possible. However, the sensitivity of the measurement is somewhat less than that of the double extraction procedure.

## *Double Extraction Procedure*

The method described below effects the isolation of the atabrine from the biological material by extraction of the free base with ethylene dichloride at pH 8.0. The latter phase is then washed with 2.5 N NaOH and the atabrine is returned as a salt to an aqueous phase of concentrated lactic acid.

## *General Considerations*

*Measurement of Fluorescence*—The intensity of atabrine fluorescence is subject to many factors. Some of these relate to the activating energy

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and others to the environment in which atabrine occurs. It has usually been considered advisable to measure the fluorescence intensity of atabrine in a weakly alkaline solution with the 365  $m\mu$  band of the ultraviolet, because of the relatively high intensity of the fluorescence obtained under these conditions. However, it has been found that the fluorescence of atabrine in acid solution is also great when the 420  $m\mu$  band of the ultraviolet is utilized as the activating energy, and that the intensity may be enhanced without any sacrifice in the stability of the fluorescence by the presence of certain acids in high concentrations. The intensity of the fluorescence under the latter conditions appears to be close to the maximal obtainable.

It must be appreciated, however, that the intensity of the fluorescence to be measured may be low, since the concentration of atabrine is so low in certain of the biological fluids, more particularly plasma. The sensitivity and precision of the estimation will therefore be conditioned, to a considerable extent, by the sensitivity and the stability of the fluorometer used in the final assay of fluorescence. Several instruments with satisfactory characteristics are now available. The No. 12 Coleman electronic photo-fluorometer has been used in these studies, since it combines these essential characteristics with simplicity of operation.

A filter system has been selected which does not result in manifest fluorescence with extracts of tissues in the absence of added atabrine. This system consists of a 2 mm. No. 5113 Corning glass filter (Coleman B4) which is used to isolate the activating energy, and a Corning No. 3385 filter (Coleman PC9) which is used to limit the transmission of the resulting fluorescent light. Advantages which are derived from this combination are the large readings obtained with atabrine fluorescence in acid solution and the ability to augment these by the addition of a non-fluorescent solute. These are achieved in the method by making the final reading in a concentrated solution of lactic acid. An added advantage of the concentrated lactic acid may be its high viscosity, which appears to be one of the factors which operate in the enhancement of atabrine fluorescence.

It has been found that the fluorescence of atabrine is partly a function of the temperature of the medium. Frequent calibration of the instrument with the standard solution results in a diminished intensity of fluorescence unless progressive heating of the solution by the ultraviolet lamp is prevented. This end is achieved by keeping both standards and samples in a water bath at room temperature before and between readings. The bath must not be below room temperature, since otherwise the separation of the small amount of ethylene dichloride which is dissolved in the lactic acid may cause turbidity. The influence of temperature on fluorescence is so great

that the manifest fluorescence may be increased 40 per cent by cooling the cuvette containing the samples and standards in an ice-salt bath. This procedure requires the addition of another ml. of lactic acid to prevent the separation of the ethylene dichloride and is not recommended as a routine procedure.

*Solvents*—Ethylene dichloride has been chosen as the organic solvent. It effects the extraction of atabrine in a highly efficient manner and at the same time does not remove many of its fluorescent degradation products from the biological sample. In addition, it has certain physical properties which recommend its use. The vapor pressure and water solubility are low and the specific gravity is high. The latter property is particularly important in that it minimizes the troublesomeness of emulsions which are commonly associated with organic solvent extractions of biological material. It must be appreciated, however, that, together with the other chlorinated hydrocarbons, ethylene dichloride is toxic to the human organism. All measurements or transfers of the solvent must, therefore, be made by automatic glass equipment.

Lactic acid is the acid of choice in the return of the atabrine to an aqueous phase, because of its efficient action in this procedure and because of the combination of properties which produce a large enhancement in the fluorescence of atabrine in acid solution (see above).

Most reagent grades of lactic acid contain fluorescent material of a foreign nature. This is routinely removed by extracting the lactic acid with ethylene dichloride in a separatory funnel. The lactic acid is then separated and shaken with a small quantity of charcoal. The charcoal is removed by filtering twice through a Buchner funnel. Small amounts of fluorescence due to foreign material in the ethylene dichloride do not usually constitute a hazard in the procedure. However, it is routine practice to remove such material with charcoal. The charcoal is removed in this case by filtering twice through a double thickness of filter paper which has been previously washed with purified ethylene dichloride. Care must be taken in both procedures to remove all carbon particles.

*Standard Solutions*—A strong solution of atabrine dihydrochloride (100 mg. per liter calculated as the free base) may be stored indefinitely in a refrigerator without deterioration. Concentrations of atabrine of 1 mg. per liter or less are used in the preparation of working standards. These are routinely prepared by diluting the concentrated atabrine solution with 0.2 M  $\text{Na}_2\text{HPO}_4$ . This precaution is essential, since the reversible adsorption of atabrine on glass surfaces in the absence of electrolyte would constitute a major error at the lower concentrations.

*Procedure*

Add 3 ml. of 0.2 M  $\text{Na}_2\text{HPO}_4$  and 30 ml. of ethylene dichloride to 1 to 10 ml. of biological material<sup>1</sup> in a 60 ml. glass-stoppered Pyrex bottle (blood is first hemolyzed with 2 parts of water). Shake vigorously for 5 minutes, preferably on a shaking apparatus. Decant into a 50 ml. centrifuge tube and centrifuge for 10 minutes at moderate speed to break the emulsion. Remove the supernatant layer by aspiration. A solid gel sometimes forms in the ethylene dichloride which may be broken by vigorous stirring with a glass rod. A second centrifugation at high speed will then produce a clean separation of the two phases. Return the ethylene dichloride solution to the original rinsed out bottle, restraining the coagulum with a stirring rod. Add an equal volume of 10 per cent NaOH and shake for 3 minutes. Remove the major portion of the sodium hydroxide solution by aspiration and transfer the remainder of the contents of the bottle to a narrow test-tube. Centrifuge for 1 minute. Remove the supernatant layer by aspiration, wash the sides of the tube with water, and remove the water by aspiration. Pipette exactly 20 ml. of the ethylene dichloride into a prepared glass-stoppered bottle,<sup>2</sup> add 1 ml. of water and 10 ml. of 85 per cent lactic acid, and shake vigorously for 5 minutes. Transfer to a prepared,<sup>2</sup> narrow 35 ml. centrifuge tube and centrifuge for 1 minute at moderate speed. Transfer at least 8 ml. of the aqueous phase to a matched

<sup>1</sup> Tissue homogenates may be simply prepared by the use of an electrically driven homogenizer. A relatively inexpensive device is distributed by the Scientific Glass Apparatus Company, Bloomfield, New Jersey. 1 or 2 gm. of tissue are added to 2 or 4 ml. of water and ground to a fine emulsion in a few minutes. Cell fragmentation is the general rule.

It is essential, because of the distribution of atabrine in whole blood, to relate the chemotherapeutic activity of atabrine to its concentration in the plasma rather than to its concentration in whole blood. This conclusion is derived from the circumstance that the concentration of atabrine in leucocytes is about 400 times that of plasma, and that variations in whole blood levels are often only a reflection of changes in leucocyte count. The unequal distribution of atabrine in whole blood requires special precautions in the preparation of plasma for analysis. The technique used is as follows: Blood is drawn with adequate amounts of oxalate as the anticoagulant. It is immediately centrifuged at 1500 R.P.M. for 15 minutes, the upper portion of the plasma removed, recentrifuged for 1 hour at 1500 R.P.M., and then carefully separated from any solid residuum. This procedure is deemed advisable in order to remove any possibility of contaminating the plasma sample with leucocytes or leucocyte fragments.

<sup>2</sup> Small blanks equivalent to 1 to 2  $\gamma$  of atabrine per liter may be obtained if the glassware has been previously exposed to air, presumably due to the accumulation of dust. The glassware for the second extraction and centrifugation must be free of water. It is rinsed with a small amount of ethylene dichloride just prior to use. These precautions are essential in the case of determinations of plasma concentrations, since these will usually range from 5 to 50  $\gamma$  per liter with the usual suppressive or curative régimes of therapy.

cuvette and determine its fluorescence in relation to a properly prepared standard. No effort is made to read the galvanometer with a greater accuracy than a quarter of a division.

The sensitivity of the fluorometer is calibrated by an atabrine standard prepared in lactic acid. The standard used in the routine estimation of atabrine during ordinary atabrine administration to humans is prepared as follows: Add 1 ml. of solution containing 0.5  $\gamma$  of atabrine to 10 ml. of 85 to 90 per cent lactic acid. A mixture of 10 ml. of lactic acid and 1 ml. of water is used for the blank setting of the instrument. The manifest fluorescence of atabrine is a linear function of its concentration in the range usually encountered when the measurement is made with a suitable fluorometer. Consequently, the calculation of atabrine concentration is by direct proportion.

*Alternate Procedure*—As noted below ethylene dichloride extracts of human blood and plasma contain little of the fluorescent degradation products of atabrine. Consequently, the alkali wash is omitted in the routine determination of atabrine concentration under ordinary conditions. The procedure is identical with that detailed above until one has produced a clean separation of the aqueous and ethylene dichloride phases after the initial extraction. A pipette is then carefully inserted below the coagulum and exactly 20 ml. of the ethylene dichloride are removed and placed in a prepared glass-stoppered bottle. 1 ml. of water and 10 ml. of 85 per cent lactic acid are added and the whole shaken vigorously for 5 minutes; the mixture is then transferred to a prepared narrow 35 ml. centrifuge tube and centrifuged for 1 minute at moderate speed. At least 8 ml. of the aqueous phase are transferred to a matched cuvette and the amount of fluorescence estimated as described in the above procedure.

### Results

Recoveries of atabrine added in known amounts to whole blood and plasma were used to assay the precision of the method (Table I). These were consecutive runs performed over a period of several months in conjunction with the routine use of the method. They give, therefore, a fair appraisal of the precision which may be expected with the routine use of the method. Table I also contains a comparable series of results obtained with the alkali wash omitted. These data indicate that atabrine added to whole blood and plasma is recoverable with good precision. A limited series of analyses indicates that equally good results are obtainable when the procedure is applied to urine.

In Table II the results obtained when whole blood and plasma are analyzed with and without the alkali wash are compared. The blood of the latter series was obtained from patients during a course of atabrine therapy.

TABLE I

*Recovery of Atabrine Added to Whole Blood and Plasma with and without NaOH Extraction. Double Extraction Method*

10 ml. samples were used.

Washed						Unwashed					
Whole blood			Plasma			Whole blood			Plasma		
Ata-brine added	Ata-brine found	Recovery	Ata-brine added	Ata-brine found	Recovery	Ata-brine added	Ata-brine found	Recovery	Ata-brine added	Ata-brine found	Recovery
$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent
1.0	1.02	102	1.0	1.02	102	2.00	1.93	97	0.50	0.49	98
	1.04	104		1.02	102		1.93	97		0.50	100
	1.04	104	0.5	0.50	100		1.90	95	0.30	0.29	97
	1.03	103		0.51	102		1.93	97		0.292	97
	1.03	103		0.50	100	1.00	0.98	98	0.20	0.18	90
	1.02	102		0.51	102		0.97	97		0.195	98
	1.03	103		0.51	102		1.01	101		0.20	100
0.5	0.48	96		0.50	100		1.03	103		0.19	95
	0.50	100		0.48	96		1.01	101		0.19	95
	0.51	102	0.2	0.18	90		0.99	99		0.197	99
	0.51	102		0.19	95		0.95	95		0.20	100
	0.51	102		0.21	105		1.04	104		0.20	100
0.25	0.26	104		0.20	100		1.00	100	0.10	0.10	100
0.3	0.30	100		0.195	98	0.50	0.50	100		0.105	105
							0.51	102		0.095	95
							0.49	98		0.095	95
							0.52	104		0.095	95
							0.47	94		0.095	95
							0.48	96		0.10	100
							0.53	106		0.102	102
							0.50	100		0.10	100
						0.20	0.20	100			
							0.195	98			
							0.20	100			

TABLE II

*Comparison of Atabrine Estimation in Human Blood and Plasma with and without Alkali Extraction Step. Double Extraction Method*

Whole blood		Plasma	
Unwashed	Washed	Unwashed	Washed
$\gamma$ per l.	$\gamma$ per l.	$\gamma$ per l.	$\gamma$ per l.
273	273	42	37
332	327	70	70
297	300	66	60
123	121	94	100
307	303	42	42
277	266	63	57
		9	10

The small difference obtained with the two procedures in this series is the basis for the judgement that an alkali wash is not essential for most routine determinations on these fluids. Similar results have been obtained on the blood and plasma of dogs to which atabrine had been administered for several months. On the other hand, the urine of both dogs and humans contains appreciable amounts of fluorescent atabrine degradation products which are extractable by ethylene dichloride. It is necessary, therefore, to include the alkali wash in the procedure when urine is analyzed.

Analyses run on single samples of blood over a period of several days invariably give highly reproducible results. This indicates that atabrine in blood or plasma is quite stable when stored in a refrigerator.

### *Comment*

The precision of the procedure is related to the absolute amount of atabrine contained in the sample rather than to its concentration. Samples of blood or plasma as large as 10 ml. need be used only when the concentration of atabrine is in the range of 30  $\gamma$  per liter or less. Actually, good precision may be obtained at considerably lower concentrations than this with no larger volume of sample, provided special precautions are taken in the matching of cuvettes and the cleaning of glassware.

It might be thought that a reasonable procedure for obviating the alkali wash in all cases is to utilize a strongly alkaline medium during the initial extraction. Unfortunately, this is not a feasible procedure, at least for whole blood or plasma. Atabrine is quite unstable in dilute solutions when exposed to strong alkali and there is a consequent loss in precision. Secondly, extraction from strongly alkaline blood or plasma results in gel formation in the ethylene dichloride phase which is broken with difficulty.

### *Single Extraction Procedure*

The single extraction procedure involves the measurement of atabrine by its fluorescence in the initial ethylene dichloride extract of the biological material. This measurement is made subsequent to the addition of acetic acid which serves to stabilize and enhance the fluorescence. The speed and simplicity of this method recommend it for most tissue analyses, even though it has somewhat lower sensitivity than the lactic acid procedure. The sensitivity of the method permits the estimation of atabrine down to 0.5  $\gamma$  with good precision. The blank is negligible in urine and organ tissues, while in plasma and whole blood the blanks are equivalent to 2 and 4  $\gamma$  of atabrine per liter. The method is therefore not recommended for ordinary use with plasma because of the low concentration of atabrine commonly observed in this fluid.



### *General Considerations*

**Measurement of Fluorescence**—A Coleman glass filter No. B<sub>2</sub> (combination of Corning No. 5113, 2 mm.; No. 3389, 2 mm.) is used to isolate the activating energy and a Coleman Filter PC9 to limit the transmission of the fluorescent light. This filter combination diminishes the sensitivity obtaining with the Corning No. 5113 filter alone, but is used to exclude the large amount of fluorescence derived from normal components of biological material.

Fluorescence measurements taken directly on ethylene dichloride extracts are theoretically less specific than those obtained in a double extraction procedure. Actually, however, only a small difference has been observed between the two procedures when applied to blood and no difference when applied to urine of dogs and humans obtained during a course of atabrine therapy. The difference in the case of blood averages 5 per cent, and may be accounted for, in part, by the blank which is only present in the single extraction procedure.

**Solvent**—Ethylene dichloride is used as the organic extractor largely because of the reasons mentioned above. An additional consideration in the present procedure is that only small amounts of interfering substances are extracted from biological material with the solvent. This reagent is highly variable in the amount of foreign fluorescent material which it contains. However, the impurities may be easily removed with charcoal unless present in excessive amounts (see above).

**Buffer**—A borate buffer of pH 11.5 is used to adjust the samples to pH 9.5 to 10 before extraction with ethylene dichloride. This is prepared as follows: To 50 ml. of 0.6 M boric acid in 0.6 M KCl add 50 ml. of 0.6 M NaOH. A blood pH of 9.5 to 10 has been selected to minimize the extraction of interfering pigments which are extractable in significant amounts from human blood or plasma at a lower pH (8.0). The error due to the extraction of pigment at the higher pH is negligible except in the rare case.

### *Procedure*

Add 1 part of borate buffer to 1 part of biological material in a 60 ml. glass-stoppered bottle.<sup>3</sup> (The blood is first hemolyzed with 2 parts of water.) Then add 15 ml. of ethylene dichloride and shake vigorously for 5 minutes, preferably on a shaking apparatus. Decant the mixture into a 50 ml. centrifuge tube and centrifuge for 10 minutes at a moderate speed to break the emulsion. Remove the supernatant aqueous layer by aspiration. A solid gel sometimes forms in the ethylene dichloride phase. This may be

<sup>3</sup> The glassware in the single extraction procedure must be scrupulously clean. The bottles and centrifuge tubes are kept in calgonite solution and rinsed just prior to use. These precautions are necessary to prevent extraneous fluorescence due to dirt.

broken by vigorous stirring with a glass rod. A second centrifugation will then produce a clean separation of the ethylene dichloride. Return the ethylene dichloride solution to the rinsed out bottle, restraining the coagulum with a stirring rod. Add an equal volume of 10 per cent NaOH and shake for 3 minutes. Remove the major portion of the sodium hydroxide solution by aspiration and transfer the remainder of the contents of the bottle to a narrow test-tube. Centrifuge for 1 minute. Remove the supernatant layer by aspiration. Wash the side of the tube with water and repeat the aspiration. Pipette 10 ml. of the ethylene dichloride directly into a cuvette containing 1 ml. of glacial acetic acid. Slight turbidity of the ethylene dichloride does not introduce an error, since it clears in the presence of acid. The reading of fluorescence is made in relation to a properly prepared standard with the same precautions as noted above.

The standard used in calibrating the sensitivity of the instrument is prepared as follows: An aqueous solution of atabrine buffered as above is extracted with ethylene dichloride and handled in the same manner as the biological sample. Ethylene dichloride is used as the reagent blank. The computation of atabrine concentration is by direct proportion.

*Alternate Procedure*—The alkaline wash of the ethylene dichloride extracts may also be omitted when the present procedure is applied in a routine manner to human whole blood and plasma, since these contain little of the fluorescent degradation products of atabrine. The procedure is the same as the one described above until a clean separation of the ethylene dichloride and water phases after the initial extraction is obtained. A pipette is then carefully inserted below the coagulum and about 10 ml. of the ethylene dichloride are transferred directly to a cuvette containing 1 ml. of glacial acetic acid. The amount of fluorescence is estimated as described in the above procedure.

### *Results*

Atabrine added to whole blood and urine is recovered with good precision as shown in Table III. The data in Table IV indicate that the estimation of atabrine concentration by the single extraction procedure yields results somewhat higher (averaging 5 per cent) than the double extraction procedure when these are applied to the blood and urine of patients during a course of atabrine therapy.

### *Comment*

The single extraction procedure is not as sensitive as the double extraction procedure. However, it may be used to advantage for many routine purposes. The lessened sensitivity results largely from the presence of a

blank which is due to the presence of extraneous fluorescent material derived from the biological sample. The quantity of this material is small and may be neglected for most purposes when the concentration of atabrine is higher than 50  $\gamma$  per liter.

TABLE III

*Recovery of Atabrine Added to Whole Blood and Urine. Single Extraction Method*

Blood			Urine		
Atabrine added	Atabrine recovered	Recovery	Atabrine added	Atabrine recovered	Recovery
$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	per cent
1	1.00	100	3	2.96	99
	1.03	103		3.08	103
	1.02	102		3.03	101
	0.98	98	2	1.97	98
	1.01	101		1.98	99
	1.04	104		2.06	103
	1.02	102	1	1.02	102
	1.02	102		1.03	103
	1.04	104		1.08	108
	1.03	103			
	1.00	100			
	1.00	100			
	0.98	98			
	0.94	94			
	0.95	95			
	0.95	95			
	1.03	103			
	0.98	98			
0.5	0.48	96			
	0.51	102			
	0.49	98			
	0.53	106			
	0.48	96			
	0.49	98			
	0.48	96			

### *Appraisal of Specificity*

The above methods appear to have adequate precision for most purposes. However, their general usefulness also depends upon the completeness with which extraneous fluorescent material is excluded in the analysis. This aspect of the problem is somewhat simplified by the absence of a significant blank in ethylene dichloride extracts as usually prepared from atabrine-free biological fluids. The degree to which the methods are specific in such a circumstance will depend upon the extent to which they exclude fluores-

cence due to the degradation products of atabrine in the final estimation of fluorescence.

The intensity of the fluorescence manifested by a solution containing an acridine is dependent in part upon the arrangement of the optical system and in part upon certain characteristics of the solution. The latter have been used to obtain additional evidence on the degree to which the recommended procedure is specific. It is not suggested that such an examination should be substituted for that utilized by Craig (1) but it does give im-

TABLE IV  
*Comparison of Single and Double Extraction Procedures*

Biological sample	Atabrine	
	Single extraction	Double extraction
Blood	$\gamma$ per l	$\gamma$ per l.
	824	794
	724	720
	933	828
	61	60
	840	819
	755	685
	106	104
	104	106
	111	104
	164	159
	588	520
	607	607
	213	205
	569	600
Urines	448	411
	1370	1410
	1930	1970
	283	274
	1990	2100
	3200	3110
	1430	1350

portant supplementary information on the specificity of the method through the use of different criteria. It also presents a general method which may be used to some advantage in a more general fashion, particularly in situations in which the amount of the fluorescent material available for study is small. The technique involves the measurement of the fluorescence manifested by a solution at a constant temperature but at various concentrations of HCl and NaOH. The variation in the intensity of the fluorescence under these conditions results from the operation of such factors as

the pH, the ionic strength, the chloride and sodium concentration, and perhaps the viscosity of the solution.

The routine procedures recommend the use of lactic acid as the final extractor of the atabrine prior to the measurement of fluorescence, or the measurement of fluorescence in the ethylene dichloride phase itself. However, essentially all of the fluorescent material which is extractable by ethylene dichloride from plasma, whole blood, or urine at a pH of 8 to 10 can be returned to an aqueous phase with any one of a number of acids, including concentrated lactic acid and 0.1 N HCl. Consequently, if the fluorescent material remaining in the ethylene dichloride phase subsequent to washing with 2.5 N sodium hydroxide has the fluorescent characteristics of atabrine, then the probability that the method has the desired specificity has been strengthened.

Purified samples of atabrine dihydrochloride and a series of other acridines were used to construct curves in which fluorescence is related to the strength of acid or alkali in the solution. The atabrine curve, so constructed, has then been used as a standard of reference for comparison with similar curves which describe the fluorescent characteristics of the material contained in ethylene dichloride extracts of the blood and urine of patients and dogs receiving atabrine.

#### *Procedure*

Each test run started with a solution of the acridine in 0.1 N HCl at a concentration of 0.5 to 1.0  $\gamma$  per ml. 1 ml. aliquots of the solution to be examined were placed in a series of fluorometer cuvettes and 10 ml. of various strengths of sodium hydroxide or hydrochloric acid were added. Fluorescence was then measured by means of the same filter system as was used in the double extraction procedure. A value of 1.0 was assigned to the fluorescence observed with the solution to which the 1.0 N hydrochloric acid had been added. The observations were performed at room temperature. A constant temperature for all samples in any run was assured by placing the cuvettes in a water bath for some time before and between readings of fluorescence. Moderate variations in the absolute temperature at which readings are made do not constitute a source of error provided the temperature is the same for all tubes in the series.

Four general types of solutions were examined. They were prepared as follows:

*Samples of pure acridine solutions* (Fig. 1) were prepared by the addition of a small amount of the acridine to 0.1 N HCl. The series of acridines selected for examination included atabrine, three relatively simple acridines, and two more complex acridines related somewhat to atabrine itself. The structural relationships of these six acridines are as follows: (1) 2-methoxy-6-chloroacridone-9, (2) 2-methoxy-6-chloro-9-aminoacridine, (3) 2-methoxy-6,9-dichloroacridine, (4) 2-methoxy-6-chloro-9(3-diamylaminopropyl)am-

inoacridine, (5) 2-methoxy-6-cyano-9(1-diethylamino-4-methylbutyl)aminoacridine, (6) 2-methoxy-6-chloro-9(1-diethylamino-4-methylbutyl)aminoacridine (*atabrine*).

Mixtures of pure acridines (Fig. 2) were prepared in order to examine

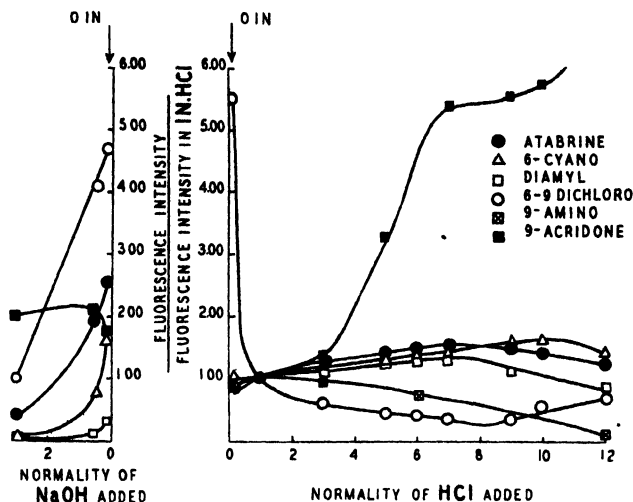


FIG. 1. Fluorescence characteristics of a series of pure acridine solutions

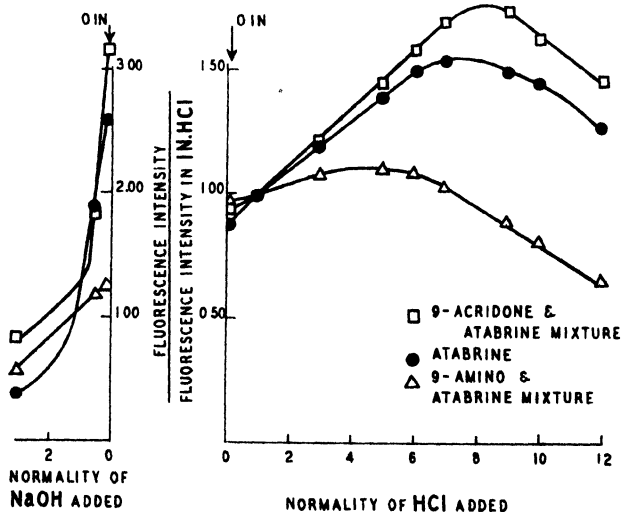


FIG. 2. Fluorescence characteristics of mixtures of pure acridines. The 9-acridone and atabrine mixture consists of atabrine contaminated with a trace of 2-methoxy-6-chloro-9-acridone-9. The latter compound is too insoluble to make a solution of known composition. The 9-amino and atabrine mixture consists of 10 per cent 2-methoxy-6-chloro-9-aminoacridine and 90 per cent atabrine.

the fluorescent characteristics of atabrine contaminated by small quantities of other acridines. These mixtures were as follows: (1) 90 per cent atabrine and 10 per cent 2-methoxy-6-chloro-9-aminoacridine, (2) atabrine contaminated with a trace of 2-methoxy-6-chloroacridone-9.

*Ethylene dichloride extracts of human and dog plasma, whole blood, and urine* (Fig. 3) in the absence of the sodium hydroxide wash. Biological samples were obtained from individuals to whom atabrine had been administered for some days. The samples were extracted with ethylene dichloride at pH 8 to 10. The fluorescent material in the ethylene di-

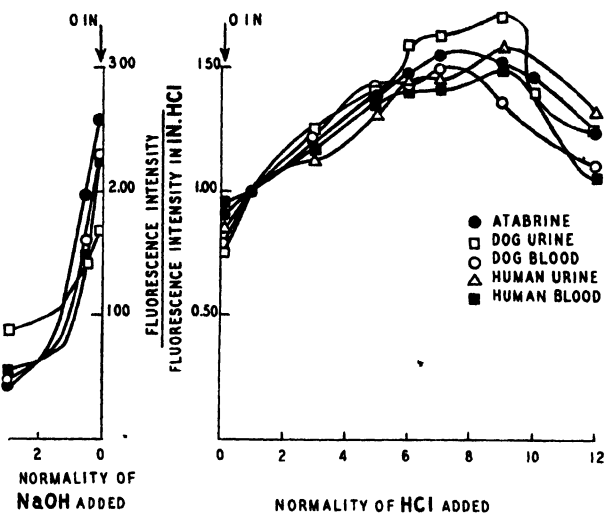


FIG. 3. Fluorescence characteristics of the fluorescent material contained in ethylene dichloride extracts of biological material in the absence of an alkaline wash. The dog and human from which the samples were obtained had received atabrine for several days previously.

chloride was returned to an aqueous phase by extraction with 0.1 N hydrochloric acid.

*Ethylene dichloride extracts of human and dog plasma, whole blood, and urine* (Fig. 4) prepared as in the method described under "Double extraction procedure." The biological samples used in this series were the same as those above. They were extracted with ethylene dichloride at pH 8 to 10; the ethylene dichloride was then extracted with an equal volume of 2.5 N sodium hydroxide and washed with water. The fluorescent material in the ethylene dichloride was then returned to an aqueous phase by extraction with 0.1 N HCl.

### Results

The results on the pure acridine solutions are summarized in Table V and Fig. 1. The data in Table V indicate that at a constant strength of acid various acridines manifest quite different intensities of fluorescence.

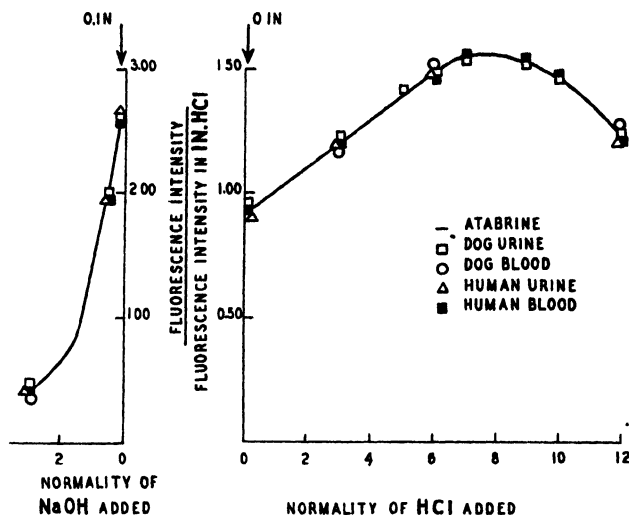


FIG. 4. Fluorescence characteristics of ethylene dichloride extracts of biological material after these extracts were washed with 2.5 N NaOH.

TABLE V

*Relative Values for Intensity of Fluorescence Manifested by Six Acridines at a Concentration of 1 Mg. per Liter in 1.0 N HCl with Standard Filter Arrangements*

Compound	Galvanometer reading
2-Methoxy-6-chloroacridone-9 (saturated solution) . .	100
2-Methoxy-6-chloro-9-aminoacridine . . . . .	400
2-Methoxy-6,9-dichloroacridine . . . . .	7
2-Methoxy-6-chloro-9(3-diamylaminopropyl)aminoacridine . . . . .	46
2-Methoxy-6-cyano-9(1-diethylamino-4-methylbutyl)-aminoacridine . . . . .	26
2-Methoxy-6-chloro-9(1-diethylamino-4-methylbutyl)-aminoacridine . . . . .	45

Furthermore, it is apparent from Fig. 1 that the fluorescence of each acridine varies in a systematic manner with variations in acid or alkali strength and that the variation is characteristic for each acridine. The curves relating fluorescence intensity to acid and alkali strength are highly reproducible.



It may be predicted from these results that the degree to which the fluorescence characteristics of a mixture of acridines will depart from the standard curve for each depends upon two factors. The first of these is the absolute magnitude of fluorescence of each of the acridines of the mixture at the standard hydrochloric acid concentration of 1.0 N. The second is the degree to which the curves describing the fluorescent characteristics of each differ, one from the other. The curves constructed from a solution of atabrine contaminated by other acridines are given in Fig. 2. Calculations performed upon data from such curves indicate that the curve observed in the case of a mixture of known composition is the simple arithmetical mean of the curves of the separate constituents.

It may be concluded from these considerations and from the curves describing the fluorescent material in the four biological extracts shown in Fig. 3 that the latter extracts contain fluorescent material with characteristics that differ from those of atabrine. It is to be emphasized that the departure of these samples from the standard curve of atabrine is not due to the manipulations of the procedure. This possibility has been excluded by the observation that atabrine added to whole blood and urine may be extracted with ethylene dichloride and then with 0.1 N HCl without change of its fluorescent characteristics. The curves derived from the biological samples do not yield information on the order of magnitude of the contaminating substance or on its nature. The variation from the normal curve may result from a small amount of a substance with a relatively high fluorescence or a large amount of a substance with a relatively low fluorescence.

The curves presented in Fig. 4 are from data which describe the fluorescent characteristics of material extracted from the same biological samples as were used in the experiments summarized in Fig. 3 with but one difference. This consisted in an alkaline wash of the initial ethylene dichloride extract, as in the analytical procedure. The data describing the characteristics of those extracts fall so closely along a curve which is characteristic of atabrine that it is unlikely for a significant amount of the fluorescence to be due to other material.

Similar results have been obtained from a series of samples of whole blood and urine derived from five patients and three dogs. It may be concluded from these data that those contaminants which cause departure from the standard curve in Fig. 3 can be removed by an alkaline wash.

#### *Comment*

Craig (1) has examined the solubility characteristics of the fluorescent material which is extractable by ethylene dichloride from blood and urine of dogs and humans during a course of atabrine therapy. The examination included the measurement of the distribution of the material and its

manifest fluorescence in a system composed of ethylene dichloride and water-methyl alcohol mixtures. The examination was made at constant temperature and constant pH, the latter being obtained by the use of a cacodylate buffer. The distribution of the fluorescent material and the intensity of the fluorescence in each of the two phases of such a system depend upon the temperature, the pH of the aqueous phase, the dissociation constants of the fluorescent material, and the phenomenon of quenching. Ethylene dichloride extracts of blood and urine contained fluorescent materials, presumably degradation products of atabrine, which diverged in their combined solubility fluorescent characteristics from pure atabrine solutions. However, it was further demonstrated that these products are soluble in strong alkali and may be quantitatively removed by washing the ethylene dichloride extracts with 2.5 N NaOH. The residual fluorescent material was then found to have solubility characteristics which are identical with those of atabrine. It was in consequence of the latter finding that the alkali wash was incorporated in the analytical procedure.

Craig's work, together with that presented above and other unpublished studies,<sup>4</sup> clearly indicates that in the metabolism of atabrine degradation products are produced, some of which are fluorescent. However, the proposed method has a high degree of specificity, because it excludes these products in the final estimation, as evidenced by both the solubility and fluorescent characteristics of the material which is present in the final measurement. This result is achieved in part by the choice of ethylene dichloride as the organic solvent and in part by washing the ethylene dichloride extract with strong alkali. One of the groups of degradation products is insoluble in ethylene dichloride and is left behind in the initial extraction. The other group is extractable by ethylene dichloride, but can be removed from this solvent by a wash with 2.5 N sodium hydroxide. The latter group is quantitatively unimportant in the case of plasma, whole blood, and the tissues of humans and dogs. Consequently, the alkaline wash may be omitted for many routine purposes. However, most urine samples contain considerable amounts of these products and an alkaline wash is, therefore, essential even for routine determinations.

#### DISCUSSION

A method for the estimation of atabrine in biological fluids and tissue should be useful in several general circumstances. It will permit the detailed study of the general pharmacology of the drug as well as the study of its specific use in the suppression and treatment of malaria. Information on the plasma concentrations of atabrine in either of the latter conditions should prove as helpful in the quantitative control of such therapy as is

<sup>4</sup> Scudi, J., unpublished observations. Bush, T. M., Butler, T. C., and Greer, C. M., unpublished observation.

information on the plasma concentration of the sulfonamides in the control of sulfonamide therapy.

Two procedures have been described for determining atabrine. The general usefulness of the more simple single extraction procedure is limited somewhat by its lesser sensitivity. The inherent specificity of the procedure appears to be as great as that of the double extraction procedure except for the presence of a small blank which precludes its use at low atabrine concentrations.

The method of choice in any situation will depend on the concentration of atabrine in the biological sample to be analyzed as well as on the size of the sample available for analysis. The single extraction procedure is advised when the concentration of atabrine is at least 50  $\gamma$  per liter and the amount of atabrine available for analysis is in excess of 0.5  $\gamma$ . It should be noted, however, that special precautions must be taken to avert errors due to the presence of extraneous fluorescence when the single extraction procedure is used in this range of concentration.

The alkaline wash is only recommended when it is important that the measurement be wholly specific. Studies concerned with the pharmacology of the drug and its distribution and excretion fall in this general category. On the other hand, it is usually unnecessary to include this step in the routine estimation of plasma atabrine concentration when the latter datum is to be used in the appraisal of atabrine therapy. The error due to such an omission will usually be less than 10 per cent.

A method, somewhat similar to the double extraction procedure, has been recently developed elsewhere as the result of independent work (2). Extensive comparisons between this and the present methods have not been undertaken. However, it is known that the method has a sensitivity of the same order as the double extraction procedure and includes in the estimation a portion of the fluorescent degradation products of atabrine. The amounts of the latter compounds are not greatly in excess of those which are included in the double extraction procedure in the absence of an alkali wash of the ethylene dichloride. It would appear from this that it was suited for the routine of clinical therapy. However, its use for other purposes is problematic at the moment. Information is not available to indicate whether the method may be modified to make it wholly specific, as is the case for the present procedure. A disadvantage of the method stems from the fact that it is not capable of modification into a more simple single extraction procedure.

#### SUMMARY

Simple precise methods are described for the estimation of atabrine in biological fluids and tissues.

Atabrine is isolated from the biological material by an extraction of the free base with ethylene dichloride at a pH of about 8. The ethylene dichloride extract is then washed free of degradation<sup>s</sup> products with 2.5 N sodium hydroxide and the atabrine is returned as the salt to an aqueous phase of concentrated lactic acid. The estimation of atabrine concentration is then made by its fluorescence in the lactic acid.

The second method for the estimation of atabrine involves the measurement of fluorescence in the ethylene dichloride phase. The speed and simplicity of this procedure recommend it for routine use when the concentration of atabrine in the biological sample does not require excessive sensitivity on the part of the method.

These methods are specific for atabrine in that they do not include the degradation products of atabrine.

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# THE ISOLATION OF VALYLVALINE FROM GRAMICIDIN HYDROLYSATES

By HALVOR N. CHRISTENSEN

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

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This communication reports the isolation of the dipeptide valylvaline from acid hydrolysates of gramicidin, as the benzoyl derivative. Hotchkiss (1) and recently Gordon, Martin, and Synge (2) have isolated *dl*-valine from gramicidin hydrolysates. The composition of gramicidin has been discussed by the above authors (1-3) and by Christensen, Edwards, and Piersma (4).

Benzoylvalylvaline (melting at 218°, corrected) was separated after benzoylation of the fraction of gramicidin hydrolysates which forms copper salts soluble in both water and methanol (Brazier (5)). The benzoylvalylvaline appeared to be optically inactive. This compound apparently has not been described before. Upon acid hydrolysis, it yielded 1 mole of benzoic acid, two carboxyl groups in addition to benzoic acid, and two  $\alpha$ -amino groups. About 90 per cent of the nitrogen was recovered as valine hydrochloride, 80 per cent as acetylvaline, and 50 per cent as *p*-toluenesulfonyl-*dl*-valine, in three separate experiments. The melting point of the acetyl derivative agreed with that recorded previously (6), and that of the *p*-toluenesulfonyl derivative with the value obtained for synthetic *p*-toluenesulfonyl-*dl*-valine (170°, corrected). This compound could not be found recorded in the literature. The melting point showed no change when the synthetic *p*-toluenesulfonyl derivative was mixed with that derived from gramicidin.

The yield of valylvaline obtained was about 1.5 per cent of the gramicidin taken when hydrolysis was carried out with boiling 16 per cent hydrochloric acid for 24 hours. When the period of hydrolysis was reduced to 6 hours, 5 and 6 per cent of the dipeptide could be separated in two cases. No valylvaline could be isolated when gramicidin was hydrolyzed for only 2 hours under the same conditions.

## EXPERIMENTAL<sup>1</sup>

*Hydrolysis and Fractionation*—Gramicidin was isolated from tyrothricin<sup>2</sup> by the following modification of the method of Hotchkiss and Dubos (7).

<sup>1</sup> Part of the microanalyses reported are by Dr. Carl Tiedcke, New York.

<sup>2</sup> Tyrothricin has been furnished through the generosity of Dr. R. J. Dubos and of Mr. Leo Wallerstein of the Wallerstein Company, Inc.

Tyrothricin was shaken with ten portions of a mixture of equal volumes of acetone and ether (U.S.P.). This extraction was performed twice more. The extract was concentrated to a thin syrup and poured into U.S.P. ether, 20 times the mass of the tyrothricin. The resulting precipitate was extracted three times with thrice its volume of a mixture of equal volumes of acetone and absolute ether. The ether and the ether-acetone solutions were combined and evaporated to dryness *in vacuo*, and the residue was crystallized from anhydrous acetone repeatedly, as described by Hotchkiss and Dubos (7). Our yields have been about 5 per cent, similar to those of Gordon, Martin, and Syngé (2). Much material of composition similar to gramicidin remained in the first acetone mother liquor.

Hydrolysis of gramicidin was carried out as described by Hotchkiss (3), a carbon dioxide atmosphere being maintained. It was found possible to add all of the hydrochloric acid during the first 2 hours of hydrolysis without causing precipitation.

At the termination of the hydrolysis period, the solvent was removed *in vacuo* and the residue repeatedly treated with water and exposed to a vacuum at 100°, to remove excess HCl. The hydrolysate in aqueous solution was then shaken with an excess of silver oxide to remove chloride. The silver was removed with hydrogen sulfide, and residual hydrogen sulfide removed *in vacuo*. The copper salts were now prepared and fractionated according to the method of Brazier (5). The fraction soluble in both water and methanol was freed from copper with hydrogen sulfide, and hydrogen sulfide was removed *in vacuo*.

*Benzoylvalylvaline*—Material obtained as just described from a hydrolysate representing 1 gm. of gramicidin was adjusted to 25 ml. and benzoylated at 0° with benzoyl chloride equivalent to three or four times the nitrogen present, the pH being maintained at about 7 (with phenol red indicator) with 4 N sodium hydroxide solution. The benzoyl chloride was added in four portions, and rapid agitation maintained until no more alkali was required. The solution was filtered, acidified with sulfuric acid, and, after a few hours at 5°, the precipitate was removed, washed and dried, and extracted with warm petroleum ether to remove benzoic acid. The residue was dissolved in boiling acetone, from which large clear monoclinic prisms separated after several hours at 5°. These were recrystallized three times from acetone. The melting point was 218°, corrected; the neutralization equivalent, calculated 320.2, found 325. No nitrogen was released by nitrous acid and only the carboxylic dissociation could be detected by electrometric titration from pH 2 to 12.

*Analysis*— $C_{17}H_{24}N_2O_4$ . Calculated. C 63.71, H 7.56, N 8.75

Found. " 63.88, " 7.81, " (Dumas) 8.80

The compound was highly insoluble in water, insoluble in petroleum ether, and soluble in alcohol and glacial acetic acid. A blue complex was formed

with alkaline cupric sulfate solution. No optical activity could be detected for the benzoylvalylvaline sodium salt in 0.3 per cent aqueous solution. Thanks are due to Dr. Paul D. Bartlett for assistance with this determination. The preparation employed as well as those studied below was obtained from gramicidin which had been hydrolyzed 6 hours. Considering the conditions of the determination, the rotation could not have been greater than  $\pm 2^\circ$ .

*Hydrolysis of Benzoyl Derivative*—12 mg. of the above compound were hydrolyzed with 0.2 ml. of 6 N hydrochloric acid in a sealed tube at  $110^\circ$  for 24 hours. About 12 hours were required completely to dissolve the material. Benzoic acid was extracted from the hydrolysate with benzene; this extract was washed once with water and titrated in the presence of 2 ml. of water to the phenol red end-point with standard alkali. 1 equivalent of benzoic acid for every 350 gm. of the benzoyl derivative (theory, 320.5 gm.) was recovered. Crystals of benzoic acid (melting at  $121^\circ$ ) were isolated upon acidification of the titrated aqueous phase.

The aqueous hydrolysate remaining after removal of benzoic acid was repeatedly taken to dryness with small portions of water to remove HCl. The residue was dissolved in 2.5 ml. of water and titrated, a glass-electrode being used. The presence of 2 equivalents each of acidic and basic groups for each mole of benzoyl derivative was shown, with pK values of about pH 2.3 and 9.5. The form of the  $\alpha$ -curve (8) did not indicate any difference in the dissociation constants of the two carboxyl groups, nor of the two basic groups, respectively. The titrated solution was washed into a 10 ml. volumetric flask and made to volume. Amino nitrogen determinations on 1 ml. aliquots showed that all the nitrogen was released in 3 minutes by nitrous acid.

1.65 mg. of the benzoylvalylvaline were hydrolyzed 2 hours in a boiling mixture of 1 ml. of acetic acid and 4 ml. of 6 N HCl. The hydrolysate was taken to dryness and washed into the Van Slyke apparatus. 0.146 mg. of N, or 101 per cent of the total N present, was recovered as amino N by the action of nitrous acid.

*Valine Hydrochloride*—A hydrolysate prepared in the same manner from 10 mg. of the benzoyl derivative was freed from benzoic acid, excess hydrochloric acid, and water. The product dissolved very readily in absolute ethanol. Addition of 5 volumes of dry ether caused precipitation of translucent crystals. These were dried at  $100^\circ$  *in vacuo* over  $P_2O_5$ . The yield, on the basis of nitrogen analysis, was 90 per cent of the N of the benzoyl derivative. Acetyltable groups, determined by a slight modification (involving measurement of about 20 c.mm. of acetylating mixture by micro pipette, and acetylating in sealed ampules) of the method of Stodola (9), were 1.05 per N atom. The analyses for C, H, and N revealed the presence of water, but were not inconsistent with the results to be anticipated



from the hydrochloride of an aminovaleric acid. No optical rotation could be detected.

*Acetyl-dl-valine*—The remaining 8 ml. of the solution recovered from the above electrometric titration were concentrated to 0.5 ml. and acetylated with acetic anhydride at 0° (10), 3 equivalents of acetic anhydride being used to increase the yield, and the danger of racemization disregarded. The solution was then acidified and the acetyl derivative was extracted by 60 volumes of chloroform used in three equal portions. 90 per cent of the N was extracted. The chloroform was removed *in vacuo*; benzene was added to the residue which dissolved but crystallized out at once. The crystals were dissolved in acetone and recrystallized by the addition of benzene. 80 per cent of the original N was recovered. The melting point was 149°, corrected. Martin and Synge (6) give 144–146° for acetyl-*dl*-valine, 157–158° for acetyl-*l*(+)-valine.

*Analysis*— $C_7H_{13}O_3N$ . Calculated. C 52.80, H 8.23, N 8.80  
Found. " 53.15, " 8.64, " (Kjeldahl) 8.93  
Neutralization equivalent. Theory 165, found 171

The two foregoing experiments established that an aminovaleric acid, apparently *dl*-valine, accounted for both of the nitrogen atoms of the benzoyl derivative of the peptide.

*p*-Toluenesulfonyl-*dl*-valine—An aqueous solution prepared by hydrolysis of the benzoyl derivative and removal of benzoic acid and excess of hydrochloric acid, in the manner already described, was treated with *p*-toluenesulfonyl chloride and alkali as described in the case of alanine by Bergmann and Niemann (11). The *p*-toluenesulfonyl derivative was crystallized twice from 30 per cent alcohol and dried 5 hours at 100° *in vacuo* over phosphorus pentoxide. 50 per cent of the N of the benzoyl derivative was recovered. *l*(+)- and *dl*-valine were converted to the *p*-toluenesulfonyl derivatives in the same manner. The melting point of the compound derived from gramicidin was 170°, corrected, that of *p*-toluenesulfonyl-*l*(+)-valine 147° (this value is given by Karrer and Veer (12)), of the *dl*-valine derivative 170°, and of a mixture of the unknown and the *dl*-valine derivative 170°. *p*-Toluenesulfonyl-*dl*-valine apparently has not been described.

This experiment proved that the aminovaleric acid was *dl*-valine.

#### DISCUSSION

The foregoing evidence for the existence of valylvaline as a portion of gramicidin provides the first available information concerning the arrangement of the amino acid residues of the polypeptide molecule. If the molecule contains two valyl residues as suggested by Hotchkiss (1), they must occur in juxtaposition. Our data do not permit conclusion as to whether all of the valine in gramicidin occurs in juxtaposition to other valyl residues.

The partial stability of this dipeptide to acid hydrolysis for 24 hours may be compared with the stability observed by Aberhalden and Vlassopoulos (13) at 37° to normal alkali. The ready cleavage of the benzoyl derivative is in agreement with their observations of the hydrolysis of 2-naphthalene-sulfonylvalylvaline and glycylvalylvaline by normal alkali at 37°. They found in general that the peptide bond of acylated dipeptides is less stable than the same bond in the dipeptides themselves. The formation of this dipeptide from gramicidin must depend not only upon its stability, but also upon the nature of the residues adjacent to it. Aberhalden and Komm (14) isolated from swine bristles, after 14 days of hydrolysis by 70 per cent sulfuric acid at 37°, a substance which appeared to be valylvaline. The rotation (+3.5°) and melting point, however, differed markedly from those found by Aberhalden and Vlassopoulos (13) for synthetic *l*(+)-valyl-*l*(+)-valine ( $[\alpha]_D^{20} = -54^\circ$ ). Fischer and Scheibler (15) synthesized *d*(-)-valyl-*l*(+)-valine, specific rotation  $-74^\circ$ .

There are three possibilities regarding the presence of racemic valine in gramicidin hydrolysates: the valine may have been racemized selectively during hydrolysis, it may be racemic originally, or *d*- and *l*-valine may co-exist at separate points in the molecule. The isolation of valylvaline indicates that if the last is the case, then the two enantiomorphs occur in contiguity in gramicidin. The apparent optical inactivity of the compound isolated contributes evidence for the existence in gramicidin of racemic valine and racemic valylvaline. The possibility that both valyl residues have been racemized during manipulation still exists, but seems small considering that the amino group of one residue has not been disengaged from the peptide link, and considering the short period of hydrolysis employed. If valine and valylvaline are present in racemic forms, gramicidin as isolated would seem to consist of isomeric compounds differing as to the configuration of the valine occurring in two specific positions in the molecule. Whether the dipeptide derivative isolated contains one or both of the possible pair of enantiomorphous valylvalines is not yet known.

#### SUMMARY

Valylvaline has been isolated as the benzoyl derivative (melting at 218°) from hydrolysates of gramicidin prepared by boiling this substance with 16 per cent hydrochloric acid for 6 or for 24 hours. The resulting amino acid mixture is subjected to fractionation as the copper salts and the fraction soluble both in water and in methanol is freed from reagents and benzoylated. No valylvaline could be isolated when the hydrolysis period was reduced to 2 hours. The benzoylvalylvaline appeared to be optically inactive. Upon complete hydrolysis, this benzoyl derivative has been shown to yield benzoic acid and 2 molecules of *dl*-valine. The valine has

been identified as the acetyl derivative and the *p*-toluenesulfonyl derivative (melting at 170°).

The implications of the presence of valylvaline in gramicidin hydrolysates have been discussed.

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# MODIFICATION OF HOLDEN'S GASOMETRIC METHOD FOR DETERMINATION OF CARBON DIOXIDE FORMED BY YEAST ACTION, AND ITS APPLICATION TO SUGARS IN VARIOUS COMBINATIONS IN BIOLOGIC MEDIA\*

By WILLIAM R. THOMPSON

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

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Holden (1) has described a method for direct estimation of fermentable blood sugar by measurement of the  $\text{CO}_2$  produced by the action of live yeast in the manometric apparatus of Van Slyke and Neill (2). We have been unable to use the special brewers' yeast favored by Holden (Fleischmann Laboratories, No. 2019) because of difficulties of supply and transportation, and have had recourse to an alternative used also by Holden, Fleischmann's bakers' yeast,<sup>1</sup> which is supplied in cakes for retail trade. In our early work, by Holden's macromethod with the timing scheme given in his addendum, we encountered great variability in blank determinations. In systematic work with a single manometric apparatus, it seemed possible to obtain satisfactory results by introduction of corrections for temperature change and time trend to the blank values, but the variability was found prohibitively large when attempts were made to use the same yeast suspension for more than one apparatus. Aeration of the suspension was suspected to be the cause, and an extended experiment showed a marked effect so produced, greatly increasing blanks and decreasing fermenting power. Accordingly, modifications were successively introduced to eliminate oxygen from the stock yeast suspension, from the other reagents, and preferably from the sample material also. A combination of washing and blanketing with nitrogen has been found most satisfactory, although fair results were obtained with stock yeast suspensions kept between layers of mercury and albolene. In the technique employed at present, Holden's method is modified as indicated below; when no differences are indicated, the two techniques are the same or are the usual procedures described by Peters and Van Slyke (2).

## *Reagents and Apparatus—*

*Suspension of bakers' yeast.* In a 500 ml. Pyrex flask provided with a

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<sup>1</sup> It is preferable to obtain fresh material directly from a Standard Brands distribution center.

glass stopper are placed 240 ml. of distilled water. Most of the dissolved air is removed by washing with nitrogen. 12 yeast cakes (about 13 gm. each) are broken up and added, nitrogen again being used to wash out most of the air. The stoppered flask is then shaken vigorously for 2 minutes. About 50 ml. of clean mercury are then introduced and a 2-hole rubber stopper is inserted which has a glass tube for delivery of nitrogen at a level just above the mercury surface, the other hole being furnished with a glass tube of 12 mm. bore and 35 mm. length. Through this short tube is inserted a 3 ml. transfer pipette provided with a skirt made from half of a rubber bulb to act as a dust shield over the hole and so adjusted that, when the pipette is in position, the tip reaches to a point slightly above the mercury surface. Nitrogen is bubbled continuously through the suspension, which is vigorously mixed with a rotary swinging motion just before any portion is removed for use. The transfer pipette is returned to position promptly.

*Citric acid reagent.* Holden's reagent, 42 gm. of citric acid and 20 gm. of NaCl per liter, is kept in a bottle or flask likewise furnished with a rubber stopper provided with a nitrogen delivery tube and a glass-lined hole for a 2 ml. pipette. A stock solution of 5-fold strength is kept for dilution and nitrogen equilibration as required.

*Pipette fillers.* It is convenient to have attached to each pipette a safety filler, described elsewhere (3), particularly if different workers use the same pipette. Fillers are useful also with the 5 ml. stop-cock pipettes for the sample.

*Antifoam reagent.* 1 volume of diphenyl ether is mixed with 5 volumes of dioxane. 3 drops are used for each fermentation (instead of 1 drop of Holden's equal portion mixture).

*Sodium hydroxide, 5 N.* This may be kept conveniently in a mercury-sealed dropping bottle of 100 ml. capacity, as described by Holmes (4), or otherwise protected from CO<sub>2</sub> absorption.

*Methyl orange.* 0.1 per cent concentration in approximately 50 per cent alcohol.

*Saturated citric acid.* About 133 gm. of hydrated acid dissolved in 100 ml. of water.

*Oxygen-free water.* The stock distilled water in the reservoir used with the apparatus is washed with nitrogen;<sup>2</sup> its use is implied in all operations with the Van Slyke-Neill chamber.

<sup>2</sup> It is convenient to have a mercury manometer with a closed end (with flow of the mercury restricted by a choke in the tube) connected with the evacuation-nitrogen access system, and the latter with a chamber and a combined blow-off safety device and mercury manometer about 20 to 25 cm. long with an open end. Leads to the stock yeast suspension, citric acid reagent, etc., may come from this chamber. Also a

### Procedure

*Preliminary Treatment*—Excessive foaming during fermentation in the Van Slyke-Neill chamber may prevent satisfactory setting of the meniscus for the first pressure reading ( $p_1$ ). For blood, Holden recommended conventional 1:10 filtrates prepared according either to Somogyi (5) or Folin and Wu (6). We have found 10-fold dilution of broths sufficient without preliminary defecation. Recently we have developed a technique of quantitative ultrafiltration (7), for removal of substances likely to cause such foaming, which permits the use of much more concentrated material. The fermentable sugar concentration should be made equivalent to a glucose concentration between 0.01 and 0.3 gm. per liter, preferably about 0.2, in the preparation. The addition of indicator and acid for adjustment is best made in the course of dilution in preparation for the next operation.

*Removal of  $\text{CO}_2$  and  $\text{O}_2$  from Sample*—The sample is so adjusted as to contain 3 or 4 drops of methyl orange per 50 ml. and enough saturated citric acid to yield a permanent pink color. 25 to 50 ml. of the preparation are placed in a 125 ml. Erlenmeyer flask provided with a rubber stopper through which is inserted a capillary glass tube bent in a right angle. This is connected with a short piece of glass tubing by means of sufficient rubber tubing to permit easy application of a pinch-cock. The glass tip serves in turn when required to connect with one of several light rubber tubes leading from a branching system the trunk of which is controlled by a 3-way stop-cock. Thus one to three flasks may be connected to the system and whirled simultaneously (as in Holden's technique for air equilibration), but the stop cock is turned at the beginning of each half minute to a vacuum line connected to one lead and then to a nitrogen line for at least half the time, the nitrogen being under slightly more than atmospheric pressure (about 100 mm. of Hg). Whirling is continued for 4 minutes, ending with nitrogen at about atmospheric pressure in the flasks, which are then clamped shut near the elbow and disconnected beyond the glass tip. The flasks are conveniently held tilted in a spring test-tube clamp for sampling.

*Sampling*—A conventional (2), rubber-tipped, 5 ml. stop-cock pipette

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lead via a mercury trap may be made to the distilled water outflow line above each Van Slyke-Neill chamber to wash the water supply in reverse (or otherwise) with nitrogen. The mercury trap prevents backflow of water when the nitrogen is shut off. A nitrogen line may be taken from a branch in this lead before the mercury trap and ending in a glass delivery tube which fits into the cup of the Van Slyke-Neill chamber with a rubber tip as on the pipettes. The glass delivery tube should extend well above the cup and be weighted by mounting upon the tube a large rubber bulb having holes at each end of the main axis, with lead shot loaded between the bulb and the axial tube.

(preferably with filler attached) is washed with nitrogen and inserted into the flask, and a sample is removed without delay or unnecessary agitation. Remnants in the delivery tip of the pipette from recent sampling need not be removed, but the pipette top is connected alternately to the vacuum and nitrogen lines twice to wash out most of the oxygen. After removal of the sample, the flask is promptly stoppered and treated likewise, but is finally whirled slightly under an atmosphere of nitrogen as before.<sup>2</sup> The preparation of the pipettes, sampling, and reequilibration of remnants in the flask may be carried out conveniently during or prior to interval *T'* mentioned below.

*Alternative Equilibration of Sample with Air*—The technique for elimination of CO<sub>2</sub> from the sample by whirling in air, as described by Holden, may be used, but blank values tend to be somewhat less stable and about 2 micromoles of CO<sub>2</sub> greater (equivalent to about 15 mm. on the manometer) than with nitrogen equilibration as just described. However, the air equilibration was used in some experiments reported below, as will be noted. It is important under these conditions to fill pipettes carefully to avoid loss of dissolved air.

*Preparation of Manometric Apparatus*—The extraction chamber of the Van Slyke-Neill apparatus is washed with air-free water containing a few drops of saturated citric acid, usually with some shaking, and then with the water alone. Finally, as much excess water is removed from the chamber and cup as is practicable by evacuation and a slow return of mercury to the top, sweeping out the water, which is removed by means of the vacuum line through a glass tube. The tip should be drawn out like a pipette and so beveled that it will tend to draw somewhat from the cup, rather than become strongly attached to the capillary tube at the base. Just before introduction of yeast, the cup is flushed with a puff of nitrogen from a delivery tube, used also in a later operation. Vacuum, nitrogen, and water delivery tubes are arranged to hang conveniently near the cup but so as not to foul each other or the apparatus when it is being shaken.<sup>3</sup>

*Removal of Preformed CO<sub>2</sub> from Yeast*—3 ml. of yeast suspension and 2 ml. of citric acid reagent are run into the prepared extraction chamber through the cup and followed by 3 drops of the antifoam reagent, without admission of air. The last of the antifoam reagent is covered with nitrogen-washed stock water, a mercury seal is made, and the cup is thoroughly

<sup>3</sup> As is recommended in general, the flexible rubber joint below the Van Slyke-Neill chamber should be made as nearly leak-proof as practicable, preferably mounted with the bore covered with a film of stop-cock grease, and with hose clamps above and below. Any air entering the chamber seems to increase respiration of the yeast, which contributes to the CO<sub>2</sub> output.

washed with water in the customary manner (2). The chamber is evacuated and shaken (about 300 complete oscillations per minute) for a period ( $T'$ ). A pair of interval timers is started from settings made so that one will ring at the end of  $T'$  and another at the end of  $T' + T$ . Unless otherwise stated,  $T' = 2$  minutes, and  $T = 10$  minutes. The mercury leveling bulb should be left in the upper ring support ready for the following operations.

At the end of  $T'$ , shaking is stopped, the chamber set upright, and about 0.5 to 1 ml. of mercury placed in the cup. Any air or water so entrapped is removed. The nitrogen delivery tube is inserted in the manner customary for rubber-tipped pipettes, the line clamp is released, and a few bubbles of nitrogen are allowed to escape. The delivery tube should be weighted enough to remain in place.<sup>2</sup>

The extracted  $\text{CO}_2$  is then ejected in the manner described by Van Slyke (8) for a manometric urease urea method and as used by Holden except that, instead of the  $\text{CO}_2$  being washed out with air, nitrogen is used and air excluded. Thus the lower cock is opened, and, as the mercury rises and fills about one-fourth of the chamber, the upper cock is opened wide, admitting nitrogen to exceed atmospheric pressure slightly. Then the upflow of mercury is restricted, the nitrogen line is clamped off, the delivery tip is removed, and upward flow is allowed to continue somewhat more rapidly. It is convenient to prevent spattering of mercury from the cup meanwhile by placing the right palm so as nearly to cover the top of the cup. The outward flow of gas is thus more uniform also, the lower cock being controlled meanwhile by the left hand. When all but 1 or 2 ml. of the gas has been expelled, flow is restricted somewhat, and the leveling bulb is held in the left hand while the remainder is ejected completely without loss of more than a drop or two of yeast mixture, or at most just a minute gas bubble is entrapped. At that moment the leveling bulb is lowered and the flow reversed until a little of the mercury from the cup enters the upper stop-cock. The lower cock is closed, the upper cock left wide open, and the bulb placed in the lower ring. Mercury may be added to the cup if required for the following operations.

The mercury surface and the exposed upper part of the cup are washed thoroughly with water, with the vacuum tube tip gradually lowered to just above the mercury. The excess water and a little mercury are drawn off to make the apparatus ready for admission of the sample.

*Simultaneous Fermentation and  $\text{CO}_2$  Extraction*—With the prepared stop-cock pipette, 5 ml. of sample are introduced in the usual way,<sup>4</sup> and the

<sup>4</sup> The rubber tip should make a good fit, and the flow seems best controlled by the pipette stop-cock, with the upper (chamber) and lower (leveling system) cocks wide open till the sample is discharged from the pipette. Then the pipette and the cham-



chamber stop-cock sealed with mercury ((2) p. 240, Fig. 30). The chamber is evacuated and shaken as before until the end of the second period ( $T$ ); i.e.,  $T' + T$  from the beginning of the first shaking interval.

*Reading of  $p_1$* —The gas volume is brought to the calibrated value (2 ml.) with care to avoid unnecessary agitation of the meniscus (*cf.* (2) p. 277), and  $p_1$  is read.<sup>5</sup> The temperature of the water jacket ( $\theta$ ) is read immediately.

*Absorption of  $\text{CO}_2$  and Reading of  $p_2$* —Then 0.5 or 1.0 ml. of 5 N NaOH is admitted into the chamber, the stop-cock sealed with mercury, the  $\text{CO}_2$  absorbed, and  $p_2$  read, all as described by Van Slyke and Folch (9). The smaller amount (0.5 ml.) of the alkali solution is used unless the sampled material contains considerable buffer.<sup>6</sup>

*Blank Analysis*—The blank sample contains per liter 5 gm. of  $\text{Na}_2\text{SO}_4$ , 60 drops of methyl orange, and 60 drops of saturated citric acid. It is equilibrated with nitrogen or air, just as is the corresponding sample material. The analysis is performed in the same manner. The resulting  $p_2 - p_1 = c$ , which may be employed in the usual way (2) if small relative to values for the corresponding analyzed samples; but as a rule it is preferable to take  $P_{\text{CO}_2} = p_2 - p_1 - c_0$  in each case (expressed in mm.),<sup>6</sup> and calculate  $\text{CO}_2$  yields as indicated below.

*Calculation of  $\text{CO}_2$  Formed*—From Table I is obtained the factor ( $10^3 \cdot F$ ) for any temperature  $\theta$ , from which the  $\text{CO}_2$  formed ( $Q'$ ) is calculated;<sup>7</sup>  $Q' = (10^3 \cdot F) \cdot P_{\text{CO}_2}$  in micromoles. Let  $C'$  be the value so calculated for

ber stop-cocks are closed, and the pipette is removed. The sample is run completely into the chamber, followed by a little mercury to seal the cock. By this procedure the pipette is guarded against contact with yeast.

<sup>5</sup> As pointed out by Holden, a strong light is needed for reading the meniscus on account of the dense yeast suspension. Preferably it should consist of a narrow beam directed from the side and slightly from behind and below the calibrated mark. A Burton Fresnel medical light, desk model (No. 1200, Burton Manufacturing Company, Chicago), remounted as convenient on a rod, with a small aperture, is an excellent source. A flash light may also be used.

<sup>6</sup> For convenience  $c_0$  is used to denote the second component of the  $c$  correction as described by Peters and Van Slyke ((2) p. 279), owing to admission of 0.5 or 1.0 ml. of alkali solution under like circumstances but with the gas pressure unaffected. It may be estimated by having both the stop-cock at the manometer top and that at the chamber top left open to atmospheric pressure during readings with a simulation otherwise of the operation of adding alkali between readings,  $p_1$  and  $p_2$ . Then  $c_0 = p_2 - p_1$ . For the apparatus we have used,  $c_0 \cong 1.0$  mm. per ml. of the alkali solution. Occasional check readings of  $p_2$  may be made after further mixing (possibly with a further addition of 5 N NaOH and appropriate correction for the altered  $c_0$ ) to guard against gross errors by failure to absorb the  $\text{CO}_2$  completely.

<sup>7</sup> These factors ( $10^3 \cdot F$ ) were obtained from those given by Holden originally calculated from the formula of Van Slyke and Sendroy (10).

a corresponding blank analysis.<sup>8</sup> Then  $Q = Q' - C'$  is the estimated  $\text{CO}_2$  in micromoles formed by the fermentation of the sample material.

*Estimation of Glucose Equivalent*—A standard glucose solution ( $S$ ) is made to contain a known glucose concentration denoted by  $G_s$  (gm. per liter) and approximately the same concentration of methyl orange and saturated citric acid as in the blank solution. It is equilibrated and treated in the same manner as the blank and the sample material. Let  $S'$  denote the micromoles of  $\text{CO}_2$  formed ( $10^3 \cdot F \cdot P_{\text{CO}_2}$ ) and  $S = S' - C'$ . Then the estimated glucose equivalent in the sample analyzed is denoted by

$$(1) \quad G = \frac{Q \cdot G_s}{S} \text{ (in gm. glucose per liter)}$$

Usually  $G_s$  is taken as 0.2 and Equation 1 holds to a fair approximation for values of  $G$  between 0.01 and 0.3, the 5 ml. of sample preparation containing from 0.05 to 1.5 mg. of glucose. This is illustrated by results obtained with glucose preparations in various concentrations, given in

TABLE I

*Factors ( $10^3 \cdot F$ ) for Conversion of  $P_{\text{CO}_2}$  to Micromoles of  $\text{CO}_2$  Formed for Various Temperatures ( $\theta$ )*

These are essentially the factors used by Holden times 1000, before introduction of any assumed yield of  $\text{CO}_2$  per mg. of glucose

$\theta, ^\circ\text{C}$	20	21	22	23	24	25	26	27	28	29	30
$10^3 \cdot F$	0.1380	0.1368	0.1357	0.1346	0.1336	0.1325	0.1314	0.1304	0.1295	0.1286	0.1277

Table II. The results are expressed in terms of  $Q/S$  and, accordingly, indicate the estimated amount of glucose in mg. present in each fermentation, as the 5 ml. of standard contained just 1 mg.

As suggested by Holden, it is preferable to have between 0.25 and 1.5 mg. of glucose in the test fermentations; dilutions should be made accordingly. A means of avoiding excessive dilution of original material is offered by the technique of quantitative ultrafiltration mentioned previously, and in association with analyses of such material (7) there is indication that refinements gradually introduced have improved the accuracy of estimation of amounts of glucose as small as 0.05 mg. (i.e., concentrations about 0.01 gm. per liter).

<sup>8</sup> Blank values ( $C'$ ) obtained with samples equilibrated with air are illustrated in Table III. In recent work (7) with universal nitrogen equilibration, the following sets of blank values ( $C'$ , in micromoles of  $\text{CO}_2$ ) were obtained on three different machines nearly simultaneously: October 19, 1942, 1.15 p.m., 1.12, 1.07, 1.13; 3.15 p.m., 1.01, 1.01, 1.00; October 20, 1942, 1.20 p.m., 0.92, 0.93, 0.92; 3.30 p.m., 0.98, 1.03, 1.03; October 21, 1942, 11.00 a.m., 1.35, 1.31, 1.24; 2.45 p.m., 1.04, 0.96, 0.99.

### Applications

*Estimation of Glucose in Presence of Maltose*—Preliminary investigation indicated that solutions of certain maltose preparations gave little  $\text{CO}_2$  of fermentation under the test conditions, and that this slight action could be eliminated by a preliminary treatment of the maltose solution with washed yeast, in the manner used for many differential tests described by Peters and Van Slyke ((2) pp. 478-480). That not all the maltose was so removed but that most of it remained to give an adequate test was demonstrated by hydrolysis of some of the supernatant after the first yeast

TABLE II

*Glucose Estimated in Fermentations by Proportion of Yield of  $\text{CO}_2$  Relative to 1 Mg. Standard*

Glucose concentrations in the sample solutions would be given by multiplying these values ( $Q/S$ ) by 0.2 =  $G_s$  in this case. Nitrogen equilibration was used for all reagents, but the samples were equilibrated with air.

Experiment No.	Actual content		
	0 100 mg	0 200 mg.	0 500 mg
	mg	mg	mg
1	0.089		
	0.078		
	0.050		
	0.058		
2	0.035	0 157	0.490
	0.053	0.175	0.504
	0.068	0.168	0.490
		0.160	
3	0.091	0.182	0.528
	0.101	0 206	0.510
	0 090		
Mean	0.071	0.175	0.504

treatment and subsequent test in the Van Slyke-Neill apparatus as above. Stark and Somogyi (11) have made more delicately controlled studies of such removal of glucose from maltose preparations, basing estimations on reducing action. Presumably, the  $\text{CO}_2$  formed under the present fermentation conditions from maltose preparations is from glucose contamination. Moreover, as indicated below, some maltose preparations appear thus to have glucose equivalent to only 2 parts per thousand of maltose content.

That glucose fermentation proceeded to sensibly the same extent regardless of the presence of large amounts of maltose was demonstrated by an experiment consisting of associated fermentation tests of solutions *nomi-*

nally as follows: solution  $\alpha = 1$  mg. of glucose per 5 ml., solution  $\beta = 25$  mg. of maltose per 5 ml., and solution  $\gamma = 1$  mg. of glucose + 25 mg. of maltose per 5 ml.

In the technique used at the time these tests were made (July 10, 1941 and July 11, 1941) nitrogen equilibration of all reagents was employed, but the sample solutions ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and blank  $C$ ) were simply equilibrated with air in the manner used by Holden. Two Van Slyke manometric machines were used simultaneously. The data are given in Table III,

TABLE III  
*Glucose Determination in Presence of Maltose*

Nitrogen equilibration was used for the yeast suspension but not for the other reagents; the samples were equilibrated with air;  $T' = 1.5$  minutes and  $T = 8$  minutes.

Approximate time (for $C'$ )	CO <sub>2</sub>				Glucose found		Recovery $\frac{\gamma - \beta}{\alpha}$
	$C'$	$(\alpha' - C')$	$(\beta' - C')$	$(\gamma' - C')$	$\beta$ solution $\frac{\beta}{\alpha}$	$\gamma$ solution $\frac{\gamma}{\alpha}$	
	micromoles	micromoles	micromoles	micromoles	mg.	mg.	mg.
12.02 p.m.	4.54						
12.24 "	4.41	9.77	0.67	10.14	0.069	1.038	0.969
2.13 "	4.24						
2.43 "	4.60	9.58	0.47	10.72	0.049	1.119	1.070
3.34 "	4.65	9.66	0.55	10.14	0.057	1.050	0.993
4.03 "	4.68						
11.34 a.m.	4.28						
12.01 p.m.	4.49	9.67	0.70	10.52	0.072	1.088	1.016
12.48 "	4.55						
2.16 "	4.41	9.23	0.51	9.91	0.055	1.074	1.018
3.06 "	4.53	9.48	0.52	10.35	0.055	1.092	1.037
4.03 "	4.39	9.36	0.61	10.43	0.065	1.114	1.049
$\bar{X}$					0.0603	1.0821	1.0217
$\bar{\sigma}_X$					0.0085	0.0304	0.0341
$\bar{\sigma}_{\bar{X}}$					0.0032	0.0115	0.0129

in which the corresponding values of  $Q$  and  $Q'$  are represented<sup>9</sup> respectively by  $\alpha$ ,  $\alpha'$ ;  $\beta$ ,  $\beta'$ ; etc. The results indicate the fair stability obtainable in blanks with two yeast suspensions each used over a period exceed-

<sup>9</sup> As a convenient aid to memory in comparisons the same symbol is used to designate the solution and the micromoles of CO<sub>2</sub> formed by fermentation of a 5 ml. sample of the solution in the prescribed test. Where ambiguity might appear the symbol is placed in brackets. Thus  $X$  is an abridged notation for  $[X] = Q_X$ . Similarly,  $X'$  is an abridged notation for  $[X'] = Q'_X$ , the total CO<sub>2</sub> formed (in micromoles), and, accordingly,  $\bar{X} = X' - C'$  as explained in the paragraph on calculation of CO<sub>2</sub> formed.

ing 4 hours.<sup>8</sup> In fact, suspensions may be used until 7 hours old, but preferably not during the 1st hour; correction should be made for the time trend in any case. The relative recovery is given by  $R = (\gamma - \beta)/\alpha = \text{mg. recovered glucose also}$ . The set of seven independent tests gave a mean  $\bar{R} \cong 1.022$  and the individual standard deviation,<sup>10</sup>  $\bar{\sigma}_R \cong 0.034$ .

About a year prior to this (July 3, 1940) a similar set of five independent tests was made on solutions of the same formula, but the reagents were not equilibrated with nitrogen and the yeast suspension was maintained simply between layers of mercury and albolene. The results gave  $\bar{R} \cong 0.979$ , and  $\bar{\sigma}_R \cong 0.022$ . The combined results (twelve tests) gave<sup>10</sup>  $\bar{R} \cong 1.004$ ,  $\bar{\sigma}_R \cong 0.036$ , and  $\bar{\sigma}_{\bar{R}} \cong 0.010$ .

*Hydrolysis Technique*—A simple technique was used to obtain total glucose after acid hydrolysis. 10 ml. of sample, or less plus water to make 10 ml., plus 1 ml. of approximately 9 N  $\text{H}_2\text{SO}_4$  are placed in a 50 ml. volumetric flask with a glass stopper converted into a valve by means of two rubber bands. The flask and contents are kept in a water bath at 98–100° for 3 hours unless another time is specified, and then cooled in air. 3 drops of methyl orange are added, enough 2 N NaOH to make the solution alkaline, then enough saturated citric acid to make the indicator a definite permanent pink (an excess of 1 or 2 drops after the first general pink coloration), and water to make the final volume 50 ml. Equilibration, with nitrogen preferably, or with air, and fermentation follow as usual in association with appropriate controls and glucose standardizations.

*Hydrolysis of Maltose*—Seven flasks, each containing 10 ml. of a solution of 1 gm. per liter of a maltose preparation,<sup>11</sup> were treated as described above, being held in the bath for  $\frac{1}{2}$ , 1, 2, 3, 4, 5, and 6 hours, respectively. Two or more fermentation determinations were made on each. Four were made on the material treated for 3 hours, and the mean  $\text{CO}_2$  yield by fermentation of these was used as a divisor ( $D$ ) to facilitate a comparison of relative results. Similarly, the *zero treatment* value was estimated by fermentation of samples of the stock solution, the result being divided first by 5 to account for the 5-fold strength and then by  $D$ . The relative results for the given time of treatment (0,  $\frac{1}{2}$ , 1, 2, 3... hours) were 0.004, 0.677, 0.877, 1.003, (1.000), 0.993, 1.010, and 1.000. Obviously, the maltose may be regarded as completely hydrolyzed in 3 hours and the hydrolyzed material, glucose, as stable under such treatment.

<sup>10</sup> Let  $\{X\}$  be any set of observations, of aggregate number  $N$ . Then the mean is denoted by  $\bar{X} = \Sigma X/N$ , and the estimate for the standard deviation for individual observations is denoted by  $\bar{\sigma}_X$ , where  $\bar{\sigma}_X^2 = \Sigma (X - \bar{X})^2 / (N - 1)$ ; the estimated standard deviation of the mean is  $\bar{\sigma}_{\bar{X}} = \bar{\sigma}_X / \sqrt{N}$ .

<sup>11</sup> This appeared from other tests to contain about 0.35 per cent of glucose and 95.4 per cent of maltose.

*Test of Hydrolysis Treatment on Glucose*—The influence of the heat treatment and of the reagents added incidentally upon later fermentation tests was investigated directly by four independent experiments of the following design.

A stock solution (*A*) containing 1 gm. of glucose per liter was prepared.

Solution *S* was a standard solution made from 10 ml. of *A*, plus 3 drops each of methyl orange and saturated citric acid, plus water to make 50 ml.

Solution *H* was obtained by following the standard directions for hydrolysis, with a 10 ml. sample of *A*.

Solution *U* was the same as *H* except that it was kept as a simultaneous control at room temperature.

TABLE IV

*Relative Glucose Content in Solutions after Hydrolysis Treatment (H) and Similar Treatment Except Heating (U) and of Each in Comparison with Standard Solution (S), Each Containing Same Original Amount of Glucose and Diluted to Same Final Volume*

The yeast suspension was kept between mercury and albolene, no nitrogen equilibrations were used,  $T' = 15$  minutes and  $T = 8$  minutes. The glucose content in the standard was 1 mg. per 5 ml

Experiment No	$\frac{H}{U}$	$\frac{H}{S}$	$\frac{U}{S}$
1	0.993	0.993	1.000
2	0.989	0.995	1.006
3	0.976	1.001	1.026
4	0.998	0.991	0.993
Mean	0.989	0.995	1.006

In each experiment each of the three preparations was made in duplicate and two or more fermentations were made on each preparation. Essentially as in the notation already adopted,<sup>9</sup> the mean estimates of  $\text{CO}_2$  so formed from these solutions were designated  $S_1$ ,  $S_2$ ,  $H_1$ ,  $H_2$ ,  $U_1$ , and  $U_2$ , respectively; in each experiment the respective means for each kind of solution were designated simply by the basic symbols *S*, *H*, and *U*, respectively; i.e.,  $2S = S_1 + S_2$ , etc. The relative results ( $H/S$  and  $U/S$ ) for each experiment are given in Table IV. Apparently neither the sodium sulfate thus introduced nor the heat treatment exerts any significant influence upon the glucose content or on the reactivity of the yeast.

*Determination of Glucose and Maltose*—These results furnished a foundation for analyses of several lots of maltose available in our laboratories, a 3 hour treatment at 98–100° for hydrolysis being adopted as standard technique. The results are given in Table V. Maltose values are ex-

pressed in terms of  $C_{15}H_{22}O_{11} \cdot H_2O$  which is equivalent in mass to the glucose obtainable on hydrolysis.

*Estimation of Fructose in Presence of Inulin*—Preliminary experiments had indicated that fructose could be estimated in the same manner as glucose, except that longer periods should be allowed for fermentation; e.g., by taking  $T = 20$  minutes, with  $T' = 1.5$  minutes as before. Further experiments were carried out to see whether the presence of inulin would affect the results. These were similar to those made upon glucose in the presence of maltose. Accordingly, three solutions were prepared nominally as follows: solution  $\alpha = 1$  mg. of fructose per 5 ml., solution  $\beta =$

TABLE V

*Estimates of Glucose and Maltose in Various Preparations, As Means of  $N_1$  and  $N_2$  Observations, Respectively*

The yeast suspension was kept between layers of mercury and albolene; no nitrogen equilibrations were used, and  $T' = 1.5$  minutes and  $T = 8$  minutes.

Maker	Grade	Lot No.	Glucose	Maltose	No. of observations	
					$N_1$	$N_2$
			<i>per cent</i>	<i>per cent</i>		
A	C.P.	602	0.85	91.2	3	3
	"	576	0.35	95.4	6	3
	Technical	1,483	5.4	86.7	2	2
	C.P.	562	0.41	96.8	2	4
B	"	33,188	0.17	95.8	3	6
	"	32,739	0.24	96.6	5	9
C	Standard	10,196	1.77	91.6	4	2
	Technical	282,794*	0.66	96.1	3	4
	"	282,794*	0.75	95.2	6	9
	"	282,794*	0.74	97.1	3	9

\* Three sets of observations on this lot were run each on a different day.

25 mg. of inulin per 5 ml., and solution  $\gamma = 1$  mg. of fructose + 25 mg. of inulin per 5 ml.

The sample solutions, including the control, were equilibrated with air but all other reagents were equilibrated with nitrogen. Ten independent sets of observations were made ( $C'$ ,  $\alpha'$ ,  $\beta'$ ,  $\gamma'$ ) as in the glucose recovery experiments<sup>9</sup> in the presence of maltose, and the respective values of  $R = (\gamma - \beta)/\alpha = (\gamma' - \beta')/(\alpha' - C')$  were calculated. The mean  $\bar{R} \cong 1.063$ ,  $\bar{\sigma}_R \cong 0.032$ ,  $\bar{\sigma}_{\bar{R}} \cong 0.010$ . Fisher's  $t$  test (12) indicates that  $\bar{R}$  is significantly different from the ideal value 1; the conventionally calculated  $t \cong 6.144$  and  $P < 0.001$ .

This small but significant tendency to greater  $CO_2$  yields from fermentation in mixtures of fructose and inulin in  $\gamma$  than the sum of the separate

yields in  $\alpha$  and  $\beta$  was probably the result of a small amount of fructose in the original inulin preparation coupled with a tendency to lower relative  $\text{CO}_2$  yields with small amounts of fructose as observed in experiments with fructose in various concentrations without inulin.

The solutions thus tested were as follows: solution  $\alpha = 1$  mg., solution  $0.5\alpha = 0.5$  mg., solution  $1.5\alpha = 1.5$  mg., and solution  $2.0\alpha = 2$  mg. of fructose per 5 ml.

The ratios of  $\text{CO}_2$  yields were denoted<sup>9</sup> by  $R$  or  $[0.5\alpha]/\alpha$ ,  $[1.5\alpha]/\alpha$ , and  $[2\alpha]/\alpha$ , respectively. In a set of six independent experiments the mean  $[0.5\alpha]/\alpha$  was  $\bar{R} = 0.453$ ,  $\bar{\sigma}_R = 0.039$ ,  $\bar{\sigma}_{\bar{R}} = 0.016$  which was probably significantly less than the ideal value (0.5) for proportional yields of  $\text{CO}_2$  from the sugar. In another experiment the solution  $0.5\alpha$  was used in pairs of fermentation tests, and the mean yield of each pair was divided by that of a companion fermentation of solution  $\alpha$ . For nine such results the mean ratio  $\bar{R} = 0.457$ ,  $\bar{\sigma}_R = 0.026$ , and  $\bar{\sigma}_{\bar{R}} = 0.0088$ . This plan was adopted to obtain greater confidence in the apparent low  $\text{CO}_2$  yield with 0.5 mg. of fructose, and Fisher's  $t$  test shows this to be statistically significant,  $t = 4.95$ ,  $P < 0.01$  (approximately 0.001).

On the other hand results with the stronger fructose solutions gave nearly ideal values as follows: for six observations of  $[1.5\alpha]/\alpha$ ,  $\bar{R} = 1.500$ ,  $\bar{\sigma}_R = 0.081$ ,  $\bar{\sigma}_{\bar{R}} = 0.033$ ; and for six observations of  $[2\alpha]/\alpha$ ,  $\bar{R} = 2.014$ ,  $\bar{\sigma}_R = 0.073$ , and  $\bar{\sigma}_{\bar{R}} = 0.030$ .

This indicates that fair results can be obtained with fructose in amounts between 1 and 2 mg. in the 5 ml. samples used for fermentation, especially if the mean of three or more fermentations is used. However, the situation is not as favorable as in the case of glucose.

*Influence of Other Sugars and Glucosides*—Preliminary investigations indicated that under the test conditions used for glucose,  $\text{CO}_2$  yields from certain other sugars are not appreciable; for example, *d*-galactose, trehalose, lactose, and melibiose. Similar results were obtained with the glucosides, salicin and esculin. Presumably glucose could be estimated in the presence of these substances as in the case of maltose; possibly they in turn could be estimated by hydrolysis.

A rough estimate was made thus of lactose in a sample of beef extract broth (*B*) supposedly containing 5 gm. of lactose per liter, but suspected of a deficiency in the sugar, with comparative results on fermentation before and after hydrolysis of a solution (*A*) containing 10 gm. of lactose per liter, with dilution to appropriate concentration in view of the expected glucose formation, and with disregard for this purpose of the *d*-galactose formed.

In terms of micromoles of  $\text{CO}_2$  formed from fermentation of 5 ml. samples, the results were as follows: *A* gave directly  $-0.04$  and  $-0.03$  (sen-



sibly none), while *B* gave only 0.22 and 0.13; 2 ml. of *A* hydrolyzed and diluted to 50 ml., as usual, gave 10.11, 9.59, and 9.57, while 4 ml. of *B* hydrolyzed and diluted to 50 ml. gave 7.75, 7.82, and 7.58.

These results indicated that such estimation is practicable, and confirmed the suspicion that the broth was low in lactose content.

Tests upon raffinose indicated appreciable but slow fermentation, perhaps as a result of hydrolysis. Sucrose gave marked fermentation as should be expected from hydrolysis by the invertase present. *d*-Mannose gave considerable yields of CO<sub>2</sub> in fermentation tests, about the same as did fructose, but not as rapidly as did glucose.

*Fraction of Theoretical Yields Obtainable with Various Techniques*—The theoretical yield from yeast fermentation is taken to be 2 moles of CO<sub>2</sub> per mole of hexose. Let *Y* denote the ratio of the actual CO<sub>2</sub> formed to the theoretical yield for the amount of hexose in the sample.

With the special brewers' yeast Holden obtained  $Y = 0.863$  and with the bakers' yeast found  $Y = 0.662$ , and indicated that longer times than 3 and 5 minutes, respectively, for fermentation gave no sensible increases. He found also that aging a mixture of citric acid with a suspension of either yeast under his conditions resulted in a rapid decline of fermentative potency.

As noted above, we have used the timing scheme outlined in Holden's addendum, which fixes *T* rather than the fermentation time precisely. However, it takes between 1 and 2 minutes for operations between the end of the preliminary extraction of CO<sub>2</sub> from the yeast in the Van Slyke-Neill chamber (end of *T'*) and the beginning of the second extraction (with simultaneous fermentation). Thus the fermentation time is about 1.5 minutes less than *T*.

Under most conditions there seems to be some practical upper limit to *Y* less than unity. In standardizations with 1 mg. of glucose, the last ten yeast suspensions used at this writing gave mean CO<sub>2</sub> yields in moles per half mole of glucose as follows:  $\bar{Y} = 0.828, 0.870, 0.897, 0.891, 0.882, 0.775, 0.796, 0.747, 0.793$ , and  $0.719$ , respectively, for  $T' = 2$  and  $T = 10$  minutes. This demonstrates the need for standardization of each yeast suspension against the sugar to be determined. Per mg. of glucose, these yields range from 8.0 to 10.0 micromoles of CO<sub>2</sub>.

It is noteworthy, also, that all these yields exceed that reported by Holden for bakers' yeast ( $\bar{Y} = 0.662$ ) and compare favorably with his value for the special brewers' yeast ( $\bar{Y} = 0.863$ ). As there is no change in the reaction mixture except a minor alteration in the antifoam reagent, which has little or no influence aside from facilitating the readings, and except for the nitrogen treatment of all reagents and sample solutions as now adopted, it would seem that the exclusion of oxygen thus promotes

fermentation, possibly through stabilization of the yeast and citric acid-yeast suspensions.

Accordingly, experiments were made with a combined yeast suspension and citric acid reagent kept as is the regular yeast suspension, washed and blanketed with nitrogen. Apparently some tendency to decreased activity may thus occur at first, but, after several hours,  $\text{CO}_2$  yields usually were found to mount to nearly the theoretical value. Thus a citric acid-yeast mixture was made in a 1000 ml. flask as follows:

To 52.8 ml. of 5-fold strength citric acid reagent were added water to make 504 ml., nitrogen to wash, then 12 yeast cakes (broken), and nitrogen to wash again. The contents were shaken 2 minutes, 50 ml. of mercury were added, and a nitrogen bubbler and sampling system was set up and maintained as in the case of the regular yeast suspension. Washing with nitrogen was continued at room temperature for 5 hours. Then the flask was fitted with a glass stopper and placed in a refrigerator (at about  $5-8^\circ$ ) overnight. The next morning the combined suspension was warmed to within  $1^\circ$  of room temperature; a clean stopper provided with the nitrogen bubbler and sampling system was set up as before and left 1 hour before use.

A 5 ml. pipette was used for transfers of such citric acid-yeast suspensions for fermentation tests, 5 ml. of the mixture being used instead of 3 ml. of the regular yeast suspension and 2 ml. of the citric acid reagent. The fermentation period was increased by making  $T = 20$  minutes; otherwise the technique was the same.

A set of fermentations and controls was run and  $C'$  estimated by interpolation to account for any trend to the time when the standard  $S'$  value was obtained;  $S = S' - C'$  was found in micromoles of  $\text{CO}_2$  formed by fermentation and divided by twice the micromolar content of glucose (2000/180.16), giving estimates of  $Y = 0.09008 \cdot S$ . In computing  $S$ , correction for the calibration<sup>12</sup> of the Van Slyke-Neill chamber at the given mark was introduced. This is the usual procedure unless only relative values are needed. Three machines were used for the fermentations, but, to rule out individual differences in blank correction which sometimes appear in such tests,  $S'$  and  $C'$  were used as obtained from the same apparatus in computing each value of  $Y$ . These were found to be as follows: 1.028, 0.980, 1.005, 0.965, 1.035, 0.947, 0.934, and 1.024; the mean  $\bar{Y} \cong 0.989$ ,  $\bar{\sigma}_Y \cong 0.039$ ,  $\bar{\sigma}_Y \cong 0.014$ . Obviously, the mean does not differ significantly from the theoretical value, unity.<sup>13</sup>

<sup>12</sup> Calibration of marks on Van Slyke-Neill chambers as well as other volumetric apparatus was made by use of an apparatus described previously (13).

<sup>13</sup> A similar set of observations was obtained with  $T' = 3$ , and  $T = 20$  minutes, and a combined citric acid-yeast suspension of essentially the same formula except that it was kept at room temperature only about  $\frac{1}{2}$  to 1 hour the 1st day, stored overnight in

This opens a possibility of attaining theoretical yields from glucose fermentation in the Van Slyke manometric apparatus and indicates much greater stability of the fermentation system in an atmosphere of nitrogen than when in contact with air as in Holden's technique. Definite reproducible methods of preparing such combined citric acid-yeast suspensions have not been established, however. The standard technique as described above, that of nitrogen equilibration of all reagents and samples with the regular yeast suspension, etc., and with  $T' = 2$  and  $T = 10$  minutes, was compared in a few tests with the same technique except that  $T$  was made equal to 20 minutes. The corresponding values of  $Y_T$  were found for pairs of fermentation tests as follows:  $Y_{10} = 0.824$  and  $0.844$ ;  $Y_{20} = 0.979$  and  $0.993$ . This indicates a good prospect of attaining theoretical yields by a simple 10 minute increase in fermentation time and further indicates stabilization of the yeast system by the use of nitrogen as described.

#### DISCUSSION

The time economy, with  $T' = 2$  and  $T = 10$ , and with three Van Slyke machines and two operators, is such that tests may be run at a rate of eight to ten per hour; one operator initiates each test and quits it after the sample has been introduced, the chamber evacuated, and the shaking started (for brevity called *starting*), and proceeds to start a test at once on the next machine, in rotation, while the other operator *finishes* the test and prepares the apparatus for the next. In similar manner one operator with two machines may first start tests on each in turn and then finish each and prepare for the next tests, completing four or more per hour.

Yields of carbon dioxide from fermentation of the standard glucose with a regular yeast suspension usually do not exhibit any great trend (seldom more than .2 or 3 per cent) during a period between 1 to 7 hours after

the refrigerator, and afterward maintained at room temperature on the nitrogen bubbler system. During the first few hours, a set of companion blanks and standard glucose tests was made, on samples equilibrated with air, two manometric machines being used simultaneously with  $C'$  and  $S'$  alternated on each. The values of  $S = S' - C'$  for these pairs gave a set of values of  $Y = 0.993, 1.044, 1.036, 1.044, 1.025, 0.993, 1.073$ , and  $0.979$  with a mean  $\bar{Y} \cong 1.023$ ,  $\bar{\sigma}_Y \cong 0.032$ , and  $\bar{\sigma}\bar{Y} \cong 0.011$ . This is in substantial agreement with the other experiment. However, the suspension was maintained thus overnight and the next day three more pairs of observations ( $C'$ ,  $S'$ ) were made, giving values of  $Y = 1.196, 1.269$ , and  $1.151$ , markedly *above* the theoretical value. It is suggested in view of these results and occasional apparent trends in such experiments to values of  $Y$  above unity, that the yield of 2 moles of  $\text{CO}_2$  per mole of glucose may be near an inflection point on a rising curve relative to potency of the fermenting system rather than to an asymptotic limit. This would be similar to a familiar situation encountered in the case of the so called saturation current in an ionization chamber.

preparation, but standardizations should be performed frequently for the best results; *e.g.*, with a set of solutions for test ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $S$ ,  $C$ ) it is a good procedure to run blanks ( $C$ ) on each machine at the beginning, then  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $S$  in order, and repeat the procedure twice (each time preferably with a different machine) and then run blanks again at the end on all machines. It is well to run blanks every 2 or 3 hours also, or even more frequently if fermentation values obtained are relatively near that of the blank or else obtain more concentrated preparations (7). One blank on each machine is usually enough when there is approximate simultaneous agreement.

Glucose *recovery* experiments have been run with broths and infusions of various sorts, with results essentially the same as in the case of the glucose-maltose solutions. However, it is best to make some such tests in connection with analyses of media or sugar mixtures under new conditions. The only consistently high yields in such addition experiments were those with fructose added to inulin, and apparently, as stated above, this was due to a systematic tendency to underestimation of small amounts of fructose such as appeared to be present in the inulin preparation. Previously, some attempts were made to demonstrate a stimulating effect of inulin without success.

Among other addition tests of this kind a glucose recovery experiment was made with a veal infusion broth (14) before addition of the glucose ( $\beta$ ) and after careful addition of the equivalent of 0.2 gm. of glucose per liter ( $\gamma$ ) in comparison with a solution ( $\alpha$ ) of 0.2 gm. of glucose per liter. Each was diluted 10-fold in preparation as usual for fermentation tests. With  $R = (\gamma - \beta)/\alpha$ , as in the other recovery experiments, three sets of values gave a mean,  $\bar{R} \cong 1.025$ , with  $\bar{\sigma}_R \cong 0.033$ , and  $\bar{\sigma}_{\bar{R}} \cong 0.019$ .

The technique has been applied to studies of sugar utilization in bacterial growth with analyses made on Berkefeld filtrates. In preparation of serum-water media with added carbohydrate for differential tests on bacteria, it has been found useful to determine the original sugar content of sera in this way, and certain infusions low in original sugar content have thus been made available for similar differential tests.

The methods may readily be modified in obvious ways to furnish a means of studying the properties of yeasts under various conditions as in the study of the influence of air under the present experimental conditions prior to or during the fermentation.

#### SUMMARY

Less fluctuation and lower blank values are obtained by modifications of Holden's method. The yeast suspensions and reagents are maintained in an atmosphere of nitrogen, with which samples are also equilibrated

preferably. It is convenient to use a 2 minute period for the preliminary extraction in the Van Slyke-Neill chamber, immediately followed by a 10 minute interval in which first the preformed  $\text{CO}_2$  is washed out with nitrogen rather than with air, the sample is introduced promptly, and simultaneous fermentation and  $\text{CO}_2$  extraction occur. Thus one or two operators may run two or three machines at a rate of four or more analyses per man hour.

Applications have been made to the determination of glucose directly in the presence of relatively large amounts of maltose and in various media, and similarly to the determination of fructose in the presence of inulin. Maltose and other sugars have been determined after hydrolysis by a simple technique.

Preliminary experiments with longer intervals for fermentation indicate that sensibly theoretical yields of 2 moles of  $\text{CO}_2$  per mole of glucose are attainable with nitrogen-protected systems.

The author wishes to acknowledge the helpful advice of Dr. Donald D. Van Slyke during the course of this work.

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# A SIMPLE DEVICE FOR QUANTITATIVE ULTRAFILTRATION IN CHEMICAL ANALYSIS\*

BY WILLIAM R. THOMPSON AND JAMES J. QUIGLEY

*(From the Division of Laboratories and Research, New York State Department  
of Health, Albany)*

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In another communication (1) a modification of Holden's method (2) for determination of sugar by direct live yeast fermentation in the Van Slyke-Neill chamber has been presented. By exclusion of air, more stable blanks were obtained and greater yields of carbon dioxide attained. Applications were made to various situations, such as glucose estimation in culture media or Berkefeld filtrates. However, if the glucose concentration in material for analysis is already low (for instance, 0.1 gm. per liter), the usual 10-fold dilution directly or in the course of defecation procedures may result in a final sugar concentration too low to permit satisfactory estimation. Otherwise excessive foaming in the extraction chamber may interfere with the adjustment of the gas volume prior to a pressure reading on the manometer. As an aid in this situation the following system for quantitative ultrafiltration was developed.

An ultrafilter consisting of a nitrocellulose membrane formed on the inside of a coarse alundum crucible<sup>1</sup> is used in a funnel as in an ordinary vacuum filtration. The funnel leads directly to a calibrated tube through a rubber stopper provided also with a delivery tube for attachment to a vacuum line. If evaporation of the filtrate is to be promoted during the filtration, a capillary tube inlet, controlled by a stop-cock or a rubber tube with a Hoffman clamp, may be adjusted so as to play a fine jet of air on the surface, and the collecting tube may be immersed in a suitable bath if temperature elevation is permissible; however, these operations are not usually required in the present application of the procedure.

The filter is washed with one or more portions of water and the washings are rejected. Several 5 ml. portions of sample material (usually three) are measured in succession into the same crucible filter, but each only after the contents of the crucible have been diminished to about 1 ml. Then

\* Presented before the Eastern New York Branch of the Society of American Bacteriologists at Troy, April 17, 1942.

<sup>1</sup> For the present purposes we have found satisfactory for use an extra coarse crucible, No. 5205,  $1\frac{1}{2} \times 1\frac{1}{16}$  inches, 35 ml. capacity, porosity R-A-766, made by the Norton Company, Worcester, Massachusetts. The crucible is supported by a rubber ring (Sargent crucible holder, size No. 3, Eimer and Amend No. 8-275) in an ordinary funnel.

two 5 ml. portions of water are likewise introduced in succession. After the volume of fluid in the crucible has been reduced to about 1 ml., the filtrate is adjusted with the aid of methyl orange and citric acid (1) and brought to volume (usually 25 ml.) by evaporation<sup>2</sup> if necessary or by addition of water as required.

The membranes<sup>3</sup> are prepared as follows:

*Nitrocellulose Sol*—Weighed amounts of nitrocellulose (parlodion) and glacial acetic acid (c.p.) are shaken intermittently until well dispersed in a bottle stoppered with a cork covered on the inside with cellophane. This may require from 2 to 4 weeks. The sol is heated to 90°, allowed to cool slowly to room temperature, and then stored in a refrigerator. About 1 day prior to use, it is removed and allowed to warm to room temperature.

*Membrane Formation*—Alundum crucibles<sup>1</sup> are thoroughly dried (having been ignited at 550–600° after any previous use). They may be stored at room temperature in a desiccator. Membranes may be prepared in several crucibles simultaneously. About 25 ml. of nitrocellulose sol are put into each crucible; the crucibles are then placed in a crystallizing dish in a vacuum desiccator which is subsequently evacuated to a pressure of 80 mm. of mercury and so maintained for about 1 hour. Air is then admitted slowly to equilibrate with the atmospheric pressure, the crucibles are removed, and their contents decanted, leaving on each only a film of nitrocellulose sol as a lining. This is coagulated in 25 per cent alcohol to form a semipermeable membrane. After 30 minutes the crucibles are transferred to a bath of running tap water for 24 hours, after which they are ready for use, but may be stored in distilled water containing a small amount of NaOCl (equivalent to 25 parts per million of Cl<sub>2</sub>).

In three preliminary experiments, membranes of different porosities<sup>3</sup> were used for media with glucose added as in typical recovery experiments. The glucose recovery was respectively 98.2, 98.5, and 102.1 per cent of the theoretical value. All filters used gave satisfactory filtrates, usually with three 5 ml. portions of original sample diluted with washings, etc., to a volume (25 ml.) to yield a final concentration equivalent to 0.6 the original; i.e., the filtrates did not induce excessive foaming in the Van Slyke-Neill apparatus with the yeast mixture following the technique described in the previous article (1). Considerable dilution (e.g., 10-fold) is otherwise often required to avoid such foaming.

An application that illustrates the possible usefulness of the technique

<sup>2</sup> After completion of filtration, evaporation may be carried out conveniently in a vacuum desiccator.

<sup>3</sup> Methods of preparing and testing graded filters from parlodion and other nitrocellulose preparations have been developed by one of us (J. J. Q.) and are discussed briefly in the published "Standard methods" of this laboratory (3).

was furnished in the case of several samples of meat infusion broths that were submitted to this laboratory with a request for glucose determination by live yeast fermentation. Except that all were made from various lots of beef heart infusion and contained 1 per cent of a peptone preparation, little was known about the formulae. Five of these broths were handled simultaneously. Fermentations were run first on 10-fold dilutions according to the regular technique (1), but inasmuch as several appeared to be too low in original glucose concentration for estimation in this manner with confidence (5 ml. of the diluted sample apparently containing about 0.05 mg. of glucose), recourse was had to the ultrafiltration technique, membranes being used which were previously prepared from a glacial acetic acid sol containing 6.5 per cent by weight of parlodion. The filtrations were completed in less than 2 hours and required little attention. The concentrations of these preparations were in each case 0.6 that of the corresponding broth (three 5 ml. portions being filtered and the filtrate made up to 25 ml.). The results obtained by fermentation are given as  $G'$  in Table I, together with those obtained on the 10-fold dilutions ( $G$ ) previously, each as glucose equivalent in gm. per liter of original broth. Analyses were made in triplicate in each case as indicated. The means for  $G$  are in fair agreement, in view of the greater variability of individual results, with those for  $G'$ . The three observations in each set are independent, each being based on a different standardization value ( $S$ ) of the yeast suspension, and each run on a different manometric apparatus, blank values obtained with the same instrument<sup>4</sup> being used. The first three samples of broth contained concentrations of glucose apparently in the range in which the ultrafiltration technique should serve to increase precision. Standard deviations for  $G'$  are uniformly less than for  $G$  here. The respective composite standard deviations for the first three broths,  $\bar{\sigma}_G \cong 0.0194$  and  $\bar{\sigma}_{G'} \cong 0.00475$  gm. per liter, give a variance ratio  $\cong 16.7$  which lies between the 1 per cent and 0.1 per cent levels of significance according to the conventional test for analysis of variance (4) for the *degrees of freedom* involved,  $n_1 = 6$  and  $n_2 = 6$ .

Thus it appears possible to obtain about ten times the sensitivity formerly claimed (5) when 10-fold dilution was employed, about 0.1 gm. per liter of original material. The low standard deviation ( $\bar{\sigma}_{G'} \cong 0.00475$  gm. per liter of broth) attained by use of the ultrafiltration technique for the first three broths is probably in part due to minor improvements described elsewhere (1), as somewhat better precision also appears to have been achieved in the use of 10-fold dilution directly ( $\bar{\sigma}_G \cong 0.0194$  gm. per liter).

<sup>4</sup> The three instruments were thus operated by two observers according to the scheme of the previous communication at a rate of about four or five observations per man per hour.



There is little advantage apparent in the case of the fourth and fifth broths, for which the glucose equivalents were about 0.25 gm. per liter; in fact there appear no significant differences in the results with regard to mean or standard deviation. The agreement substantiates preliminary tests on glucose recovery in indicating recovery of essentially all the glucose in the ultrafiltrate.

Furthermore, a sixth broth was tested in like manner after ultrafiltration and in the equivalent concentration by simple dilution (6:10). The former gave  $100G' = 160, 162, \text{ and } 157$  gm. per liter, without difficulty, whereas the latter procedure yielded, respectively, 1 and 2 cm. of foam when the

TABLE I  
*Estimated Original Glucose Equivalent*

The values are given in gm. per liter. 10-Fold diluted samples were used to obtain  $G$  and ultrafiltrates six-tenths of the original concentration for  $G'$ ;  $X = G$  or  $G'$ .

	Test No.	Broth 1	Broth 2	Broth 3	Broth 4	Broth 5
$G$	1	0.119	0.102	0.053	0.263	0.270
	2	0.108	0.143	0.082	0.253	0.281
	3	0.129	0.146	0.092	0.256	0.258
Mean, $\bar{X}$		0.119	0.130	0.076	0.257	0.270
S.D., $\tilde{\sigma}_X$		0.011	0.025	0.020	0.005	0.012
$G'$	1	0.137	0.143	0.089	0.260	0.275
	2	0.132	0.141	0.091	0.248	0.273
	3	0.134	0.129	0.087	0.243	0.247
Mean, $\bar{X}$ .		0.134	0.138	0.089	0.250	0.265
S.D., $\tilde{\sigma}_X$		0.003*	0.008*	0.002*	0.009	0.016

\* The composite standard deviation estimated for  $G$  on the first three broths is 0.0194 gm. per liter, whereas that for  $G'$  correspondingly is only 0.00475.

operator was attempting to make the first readings in two instances and no foam in another test; the test without foam gave  $100G = 159$ , and the others, in spite of the foam, gave  $100G = 162$  and  $152$ , respectively. Of course no reliance could be placed upon readings under the latter conditions, but the results are in general agreement with Holden's findings (2) with 10-fold diluted blood which foamed excessively but which gave about the same mean values as the filtrates after protein removal.

#### SUMMARY

A device for quantitative ultrafiltration is described. It is used to avoid excessive dilution while permitting the preparation of filtrates which will

not foam excessively in the Van Slyke-Neill chamber during the estimation of sugar by direct live yeast fermentation, foam-producing substances of large molecular size such as proteins being retained by the ultrafilter. Other applications are suggested in which separation may be based upon differences in molecular size.

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## LETTERS TO THE EDITORS

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### SYNTHESIS OF TRYPTOPHANE FROM INDOLE AND SERINE BY NEUROSPORA

Sirs:

Evidence has been obtained demonstrating that serine is concerned in the synthesis of tryptophane by the ascomycete *Neurospora crassa*, apparently through a direct reaction with indole. It has been suggested that indole<sup>1,2</sup> and anthranilic acid<sup>2</sup> are intermediates in the synthesis of tryptophane by certain bacteria.

The disappearance of indole was followed colorimetrically in solutions containing indole and various other substances. These solutions were incubated with shaking in the presence of 3 day-old *Neurospora* mycelia. The addition of *dl*-serine permitted a complete and rapid disappearance of indole. Equivalent amounts of pyruvic acid, glyceraldehyde,  $\alpha$ - or  $\beta$ -alanine, phosphoglyceric acid, Cori ester, and sucrose had no effect on the reaction. Within a range of 10 to 50 mg. of *dl*-serine per 50 cc. the rate of indole disappearance was a function of the concentration of serine.

Colorimetric determinations of tryptophane in solutions after the disappearance of indole indicated that tryptophane was formed. This was proved by isolation. After 48 hours incubation of 50 mg. of indole, 500 mg. of *dl*-serine, and *Neurospora* mycelium (1 gm. of dry weight), the solution contained approximately 40 mg. of tryptophane (45 per cent of theoretical). This was precipitated from the concentrated filtrate with  $\text{HgSO}_4$ , recovered from the precipitate, acetylated, and crystallized. Approximately 15 mg. of crude acetyl-*dl*-tryptophane were obtained. After recrystallization, the product had the correct melting point (204–205°) and showed no depression when mixed with an authentic sample.

Analysis— $\text{C}_{13}\text{H}_{15}\text{N}_2\text{O}_3$ .	Calculated.	C 63.10, H 6.12, N 11.34
	Found	" 63.74, " 5.94, " 11.73

Department of Biology  
Stanford University, California

E. L. TATUM  
D. M. BONNER

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<sup>1</sup>Fildes, P., *Brit. J. Exp. Path.*, **22**, 293 (1941).

<sup>2</sup>Snell, E. E., *Arch. Biochem.*, **2**, 389 (1943).



# STUDIES ON THE PHOSPHOROLYSIS OF SUCROSE

By MICHAEL DOUDOROFF

(From the Department of Bacteriology, University of California, Berkeley)

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The recent demonstration of a phosphorolytic breakdown of sucrose (1, 2) by two quite different types of bacteria makes it seem possible that such a process may be of relatively wide-spread occurrence in nature. The phosphorolytic reaction has, to date, been studied only with intact cells (2) or with dried bacterial preparations (1). Little information about the phosphorolytic enzyme can be obtained from experiments on such systems because of other concurrent reactions which they catalyze. Although dry preparations of *Pseudomonas saccharophila* do not cause further transformations to any extent of the glucose-1-phosphate and fructose arising from sucrose breakdown, they exhibit a fairly strong invertase activity, which makes quantitative studies of the phosphorolysis and synthesis of sucrose impossible.

In the present paper, a method of extracting and treating such dry cell preparations is described, which permits not only the concentration of the phosphorolytic enzyme but also the virtual elimination of undesired hydrolytic side reactions. With preparations obtained by this method, a number of exploratory experiments dealing with the properties of phosphorylase have been carried out.

## EXPERIMENTAL

For the present studies, the preparation of dry bacteria and the estimation of reducing sugar, inorganic phosphate, glucose-1-phosphate, and sucrose were carried out as described in the previous paper (1).

To evaluate the phosphorylase and invertase activities in variously treated bacterial preparations, it was found convenient to establish an arbitrary unit for each enzyme. 1 unit of phosphorylase was defined as the amount of enzyme catalyzing the esterification of  $10^{-6}$  equivalent of inorganic phosphate in 20 minutes at 30° when dissolved in a M/30 Sørensen phosphate buffer, pH 6.64, containing 0.1 M sucrose. The basis for the establishment of such a unit was the observation that, under the conditions described, the rate of esterification is almost proportional to the amount of enzyme preparation used, provided the activity does not exceed 8 units per ml. It is obvious that, for measurement of activity, and for experiments on rates of phosphorolysis, the preparations had to be so diluted as to give a reaction approximating the zero order for the

duration of the experiment. The relation of enzyme concentration to the formation of glucose-1-phosphate is shown on Fig. 1. The preparation used for this experiment had been partially freed of invertase by methods to be described below. The phosphorylase activity of the dry cell preparations of bacteria grown with sucrose as substrate was found to vary from about 600 to 700 units per gm. Bacteria grown with trehalose as substrate contained almost no detectable phosphorylase (less than 10 units per gm., dry weight).

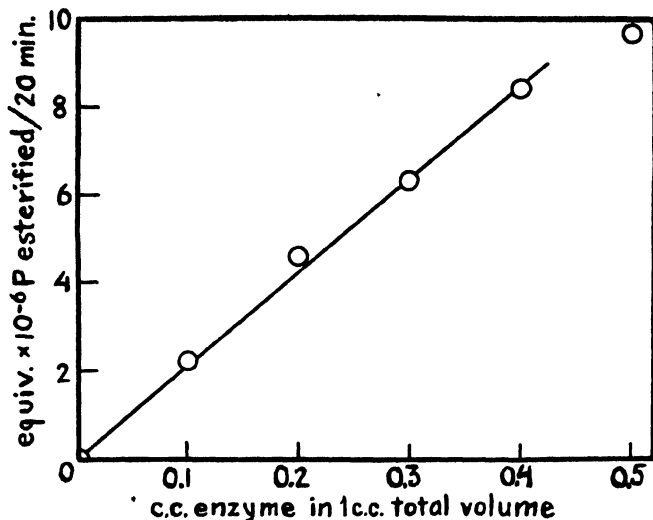


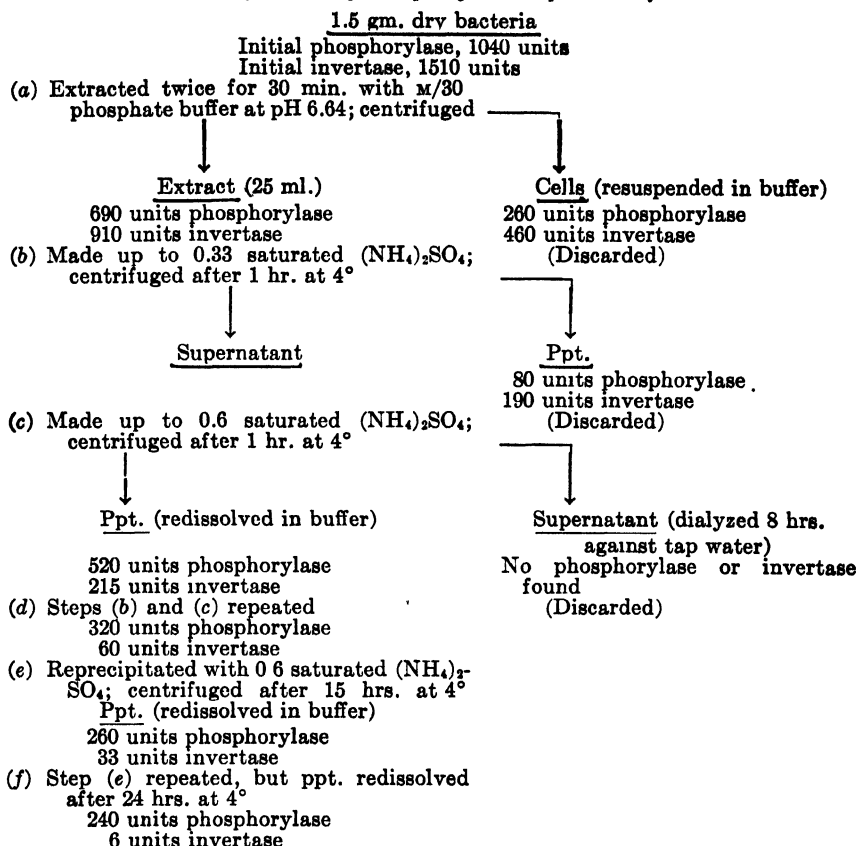
FIG. 1. Rate of phosphorolysis with different enzyme concentrations. The enzyme preparation was freed of invertase and diluted to 1 ml., with a final concentration of 0.1 M sucrose and M/30 phosphate buffer at pH 6.64.

1 unit of invertase was arbitrarily defined as the amount of enzyme catalyzing the inversion of  $10^{-6}$  mole of sucrose under the same conditions as those employed for the determination of phosphorylase activity. The reducing sugar arising from inversion was calculated from the observed increase in reducing value corrected for the theoretical increase due to phosphorolysis.

*Preparation of Phosphorylase*—The enzymes responsible for both phosphorolysis and hydrolysis of sucrose could readily be extracted from the dry cells with phosphate buffer at pH 6.64. Subsequent treatment of the extract with ammonium sulfate in the manner outlined in the accompanying diagram gave rise to preparations which contained from a third to a half of the original phosphorylase activity, but very little invertase and phosphatase. The chief loss in phosphorylase activity appeared to occur in

the early stages of the fractionation, and reprecipitated material remained active for several days in 0.6 saturated  $(\text{NH}_4)_2\text{SO}_4$  at  $4^\circ$ . The invertase was apparently inactivated by prolonged contact with 0.6 saturated  $(\text{NH}_4)_2\text{SO}_4$ , since it could not be recovered from the supernatant liquid after precipitation of the active material. Although the salting-out method

*Method Used for Freeing Phosphorylase Preparation of Invertase*



made it possible to obtain very active preparations of phosphorylase, no studies were made of specific activity, nor was any attempt made at purifying the enzyme because of the lack of facilities for obtaining sufficient material. For the experiments to be reported below, the general procedure described in the diagram was followed. After two fractionations between 0.33 and 0.6 saturated ammonium sulfate, several precipitations at 0.6 saturation were made throughout a period of 2 or 3 days during which



the preparation was stored in the ice box. Any denatured, insoluble proteins were removed by centrifugation, and a solution of the desired activity was prepared by dilution with buffer or distilled water.

*General Properties*—The phosphorylase preparations thus obtained could be dialyzed against running tap water (but not distilled water) for 18 hours with but slight loss of activity as compared with controls left in buffer at the same temperature for the same period of time. The relatively good recovery of activity after repeated reprecipitation and dialysis of the material made it seem likely that no coenzyme is involved in the reaction. It will be recalled that muscle phosphorylase requires adenylic acid, while no coenzyme appears to be needed for the action of potato phosphorylase (3, 4). Data presented in Table I, Experiment 1, indicate that the rate of phosphorolysis by a reprecipitated and dialyzed preparation was not

TABLE I  
Rate of Phosphorolysis

Experiment No.	Temperature	Additions	P esterified per 20 min per ml * ( $\pm 0.2$ )
	°C.		$mM \times 10^{-3}$
1	30	None	5.4
1	30	Heat-inactivated bacterial extract	5.3
1	30	m/300 adenylic acid	5.5
2	20	None	1.8
2	30	"	3.6
2	40	"	6.5

\* The enzyme was allowed to act 20 minutes at 40°, 40 minutes at 20° and 30°.

materially affected by the addition either of muscle adenylic acid or of extracts of cell suspensions.<sup>1</sup> No addition of sucrose was required to initiate the synthesis of this sugar from glucose-1-phosphate and fructose by dialyzed preparations. The phosphorolytic synthesis of starch with purified muscle and potato enzymes requires the addition of catalytic amounts of polysaccharide.

The phosphorylase activity of unprecipitated bacterial extracts was destroyed to the extent of about 55 per cent in 10 minutes at 55°, while the invertase activity of the same preparation was diminished by only about 27 per cent in the same period of time. The  $Q_{10}$  for phosphorolysis was found to be close to 2 between 20–40°, as may be seen from Table I, Experiment 2. Muscle phosphorylase shows an unusually low temperature co-efficient in the same temperature range (3).

The rate of phosphorolysis by invertase-free preparations was found to be

<sup>1</sup> Adenylic acid was kindly supplied by Dr. Herman Kalckar.

practically the same between pH 6.4 and 7.0, but to fall off rapidly below pH 6.0. With preparations kept below pH 5.6, the activity decreased with time, indicating a gradual inactivation of the enzyme. If this inactivation was not too great, the full activity could be recovered by neutralization, but below pH 5.2 the enzyme was rapidly destroyed at 30°.

*Phosphorolytic Reaction*—The general conclusions which were drawn from observations on the phosphorolytic activity of crude suspensions of dry bacteria (1) could be corroborated by similar experiments with phosphorylase preparations relatively free of invertase. The results of one such experiment are presented in Table II. The phosphorylase was allowed to act on approximately 0.1 M sucrose and M/30 phosphate in a phosphate-bicarbonate-CO<sub>2</sub> buffer system at pH 6.5 at 30°. It will be noted that some residual invertase activity remained in this preparation, as is indicated by

TABLE II  
*Phosphorolysis of Sucrose*

Time	mm × 10 <sup>-3</sup> per 3 ml.		
	Glucose-1-phosphate (a)	Reducing sugar formed (as hexose) (b)	Sucrose decomposed $(a) + \frac{(b) - (a)}{2}$ (c)
<i>min.</i>			
40	57.6	59.1	58.3
80	89.4	93.0	91.2
120	99.9	109.8	104.8
160	100.8	116.4	108.6
200	100.8	120.9	110.8

the production of reducing sugar in excess of glucose-1-phosphate. Any dephosphorylation of glucose-1-phosphate which had been formed would appear as inversion, since glucose and fructose would arise in equimolar ratio without apparent phosphate uptake. Hence, the quantity of sucrose decomposed may be computed from the amounts of glucose-1-phosphate and reducing sugar formed, as shown in Table II. The quantity of fructose present at any given time would be equal to the amount of sucrose which had disappeared.

Table III shows the action of phosphorylase on a mixture of glucose-1-phosphate and fructose. With these substrates, it is impossible to correct directly for side reactions involving glucose-1-phosphate or to compute the exact amount of fructose utilized. A small amount of hydrolysis of the ester would alter considerably the apparent ratio of fructose to glucose-1-phosphate used in the synthesis of sucrose, since not only a greater production of phosphate, but also a smaller disappearance of reducing sugar, would

be observed. This difficulty could be overcome by correcting the observed changes in phosphate and reducing sugar values for changes found in a control experiment, in which the preparation was allowed to act on glucose-1-phosphate in the absence of fructose. Since the phosphorylase preparations showed very little action on glucose-1-phosphate, such corrections were relatively small. The discrepancy appearing in Table III between the phosphate liberated and reducing sugar utilized may be attributed not only to experimental error, but also to slow hydrolysis by the residual invertase of the sucrose as it is formed.

A number of experiments were conducted to determine the equilibrium concentrations of the reactants under selected conditions. Fairly potent phosphorylase preparations were allowed to act on mixtures of sucrose and

TABLE III  
*Synthesis of Sucrose*

Enzyme preparation with approximately 0.8 M glucose-1-phosphate, 0.125 M fructose, 0.1 M NaHCO<sub>3</sub>, in CO<sub>2</sub> atmosphere at 30° and pH 6.6 to 6.7. Phosphate and reducing sugar values corrected for control values obtained with glucose-1-phosphate alone. Sucrose determined with the aid of invertase.

Time	M eq $\times 10^{-2}$ per ml.		
	Total inorganic P liberated from glucose-1-phosphate	Total reducing sugar utilized	Total sucrose formed
<i>hrs.</i>			
2	1.2	1.3	1.3
4	1.5	1.3	1.6
6	1.6	1.4	1.7

phosphate, with and without added fructose, and of glucose-1-phosphate and fructose. All experiments were performed at 30° and an effort was made to attain in different series final pH values of 6.6 and 5.8, respectively. Reactions were allowed to proceed until the change in inorganic phosphate concentration over a period of 1 hour had decreased to less than the value of the experimental error. The total time of incubation was usually between 7 and 10 hours. The pH, inorganic and esterified phosphate, reducing sugar, and sucrose were then determined. The fructose concentration was calculated, as explained earlier, by subtracting from the reducing sugar values the glucose formed by inversion and phosphatase activity. The procedures used in several selected experiments are outlined below, and the results are presented in Table IV. When inorganic phosphate was added, Sørensen KH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub> buffers were used. Glucose-1-phosphate solutions were prepared by adjusting the potassium salt to pH 6.3 to 6.5 with acetic acid. Bicarbonate-CO<sub>2</sub> or citrate buffers were used

in addition to the phosphate system. A tentative equilibrium constant for phosphorolysis under each set of experimental conditions was computed as

$$K = \frac{(\text{sucrose})(\text{inorganic phosphate})}{(\text{glucose-1-phosphate})(\text{fructose})}$$

In Experiments 1 and 2, phosphorylase preparations were incubated with approximately M/15 sucrose and with approximately 0.05 M and M/30 phosphate respectively in 0.1 M sodium bicarbonate in an atmosphere of CO<sub>2</sub> at 30°.

TABLE IV  
Concentration of Reactants at Equilibrium

Experiment No.	Initial constituents added	Final pH	Concentration at end, mm × 10 <sup>-3</sup> per ml.				K value
			Inorganic P	Sucrose	Glucose-1-phosphate	Fructose	
1	Sucrose, phosphate	6.6	5.9	17.9	44.9	45.7	0.052
2	" "	6.6	6.5	19.6	46.0	48.0	0.058
3, a	" " "	6.6	3.76	37.2	40.3	63.1	0.055
4	" " fructose	6.6	3.8	39.5	22.4	123.5	0.054
5	Glucose-1-phosphate, fructose	6.6	18.6	15.8	66.4	88.9	0.050
6	" " "	6.6	20.0	17.0	62.4	112.5	0.049
7, a	" " "	6.6	18.9	12.9	65.1	78.0	0.050
3, b	Sucrose, phosphate	5.8	5.26	38.2	36.3	56.4	0.098
7, b	Glucose-1-phosphate, fructose	5.8	20.3	17.7	61.3	70.0	0.084
8, a	Sucrose, phosphate	5.8	7.76	9.7	28.9	29.7	0.088
8, b	" " "	5.8	3.12	36.3	34.0	35.0	0.095

In Experiment 3, the initial sucrose and phosphate were approximately 0.1 M and M/23 respectively in 0.1 M bicarbonate-CO<sub>2</sub> buffer at 30°. A fraction of the above mixture (Experiment 3, a) was incubated under these conditions throughout. Another portion (Experiment 3, b) was removed after 3 hours, acidified with citric acid, and maintained thereafter at pH 5.8 in the absence of CO<sub>2</sub>.

Experiment 4 was similar to Experiment 1, except that the initial phosphate concentration was approximately 0.025 M, and 0.1 M fructose was added to the mixture at the start.

In Experiments 5 and 6, phosphorylase preparations were incubated with approximately M/12 glucose-1-phosphate and with 0.1 M and 0.125 M fructose respectively in 0.1 M bicarbonate in an atmosphere of CO<sub>2</sub>.

Experiment 7 was initially like Experiment 5, except that M/11 fructose

was used. A portion (Experiment 7, *a*) was incubated under the same conditions throughout. Another portion (Experiment 7, *b*) was removed after 3 hours, acidified with citric acid, and maintained at pH 5.8 thereafter.

In Experiment 8, phosphorylase preparation was incubated with approximately 0.05 M sucrose, M/22 phosphate, and 0.1 M bicarbonate in an atmosphere of CO<sub>2</sub>. After 4 hours, it was acidified with citric acid to pH 5.8 in the absence of CO<sub>2</sub>. More sucrose was added to one fraction (Experiment 8, *a*) and an equivalent amount of water to another (Experiment 8, *b*). Both aliquots were maintained at pH 5.8 thereafter.

It will be seen from Table IV that, at a given temperature and pH value, an apparent equilibrium constant may be derived for the phosphorolytic process. At equilibrium, the quantity of the products of phosphorolysis is great compared with that of synthetic products. Furthermore, the equilibrium constant changes with pH in a manner similar to that observed with muscle phosphorylase (5). The limitations of the methods employed for the quantitative estimation of the various components allowed for only a fair degree of accuracy in the determination of the constants, and did not encourage a more thorough study. A greater refinement of techniques would be desirable for a thorough investigation of the kinetics of the reaction.

*Specificity*—The enzyme appears to be quite specific for sucrose. No phosphorolysis could be detected with raffinose, trehalose, maltose, glycogen, or starch as substrates. Furthermore, there was no evidence that other sugars or phosphate esters could replace fructose in the reaction with glucose-1-phosphate. This observation is of interest, since it makes doubtful the possibility suggested earlier (1) that, in the normal metabolism of sucrose by the bacterium, the disaccharide may act first as a phosphate acceptor, being then phosphorolyzed to 2 molecules of hexose phosphate. The relative amounts of inorganic phosphate split off from glucose-1-phosphate as well as from mixtures of glucose-1-phosphate with fructose-6-phosphate, fructose diphosphate, glucose, and fructose respectively are shown in Table V. It will be noted that the production of inorganic phosphate from mixtures of esters does not exceed that attributable to their hydrolytic decomposition.

*Inhibition of Phosphorylase*—The rate of phosphorolysis of sucrose was found to be practically unaffected by the addition of 0.2 M sodium fluoride (see Table VI, Experiment 1). Furthermore, the presence of raffinose, trehalose, maltose, mannose, or galactose did not materially influence the rate (Experiments 3 and 4). Glucose, on the other hand, was found to have a strong inhibitory effect on the reaction (Experiment 5). That glucose competes with sucrose for the enzyme appears likely from the fact that the inhibition can be greatly reduced by increasing the sucrose concentration (Experiment 6). As might be expected, glucose also interferes

TABLE V

*Action of Phosphorylase on Various Substrates*

Substrates supplied in approximately 0.015 M final concentration, at pH 6.5 (adjusted with acetic acid), to phosphorylase preparation containing 0.1 M NaHCO<sub>3</sub> in an atmosphere of CO<sub>2</sub>.

Substrate 1	Substrate 2	Inorganic P liberated, $\text{mM} \times 10^{-3}$ per 2 ml. after 60 min. at 35°
Glucose-1-phosphate	None	0.3
"	Glucose	0.3
"	Fructose-6-phosphate	0.3
"	Fructose diphosphate	0.4
"	Fructose	4.5
Fructose-6-phosphate	None	0.2
Fructose diphosphate	"	0.3

TABLE VI

*Inhibition of Phosphorolysis*

Experiment No	Initial concentration		Additions	P esterified per ml. in 20 min.  $\text{mM} \times 10^{-3}$
	Sucrose	Phosphate		
1	M/9	M/30	None	5.3
	M/9	M/30	M/5 NaF	4.8
2	M/9	M/30	None	3.4
	M/9	M/30	M/150 phlorhizin	3.5
3	M/12	M/30	None	4.4
	M/12	M/30	M/12 raffinose	3.9
4	M/6	M/30	None	7.8
	M/6	M/30	M/4 trehalose	7.8
	M/6	M/30	M/4 maltose	7.8
	M/6	M/30	M/4 mannose	7.9
	M/6	M/30	M/4 galactose	7.5
5	M/8	M/32	None	6.2
	M/8	M/32	M/32 glucose	3.2
	M/8	M/32	M/16 "	2.2
	M/8	M/32	M/8 "	1.1
	M/8	M/32	M/4 "	0.7
6	M/8	M/32	None	5.2
	M/8	M/32	M/32 glucose	2.3
	M/4	M/32	None	5.1
	M/4	M/32	M/32 glucose	4.5
	M/8	M/16	None	5.4
	M/8	M/16	M/32 glucose	2.6

with the reaction in the other direction, between glucose-1-phosphate and fructose. A decrease of this inhibition could be obtained by increasing the concentration of glucose-1-phosphate, but this decrease was less pro-

nounced than that effected by sucrose in the opposite reaction. Glucose is known to interfere with the action of muscle phosphorylase by competition with glucose-1-phosphate (5).

An unusual inhibitory action was observed with phlorhizin, in that the initial rate of reaction appeared to be decreased in one direction only. Thus, the addition of  $M/400$  to  $M/150$  phlorhizin to preparations acting on sucrose and phosphate never decreased the rate of phosphorolysis (Table VI, Experiment 2). In some cases, in fact, a slight increase in rate, usually within the experimental error, was observed. With glucose-1-phosphate and fructose as substrates, on the other hand, phlorhizin in similar concentrations caused a 10 to 25 per cent decrease in the initial rate of dephosphorylation. The per cent inhibition decreased as the reaction was allowed to proceed further, so that at equilibrium the concentration of reactants was practically identical with that found in the absence of phlorhizin. No reaction between phlorhizin and inorganic phosphate could be detected, nor could the decreased rate of inorganic phosphate liberation be attributed to an inhibition of processes other than that catalyzed by the phosphorylase, since no effect of phlorhizin could be observed on phosphate liberation from glucose-1-phosphate in the absence of fructose. A possible explanation for this anomalous inhibition might be that the synthetic product derived from glucose-1-phosphate and fructose is not sucrose, but an isomer of this sugar, and that phlorhizin interferes with its formation, but not with the breakdown of sucrose. The observation that phlorhizin does not displace the equilibrium between the reactants would not be incompatible with this explanation. Although no further attempts have as yet been made to identify positively the synthetic sucrose, the data presented in the previous paper (1) seem sufficiently convincing to make such an explanation improbable.

#### SUMMARY

1. Extraction of dry cells of *Pseudomonas saccharophila* with phosphate buffer and subsequent treatment of the extract with ammonium sulfate make it possible to obtain active preparations of sucrose phosphorylase relatively free of invertase and phosphatase.

2. No coenzyme appears to be necessary for the phosphorolytic process, nor does the synthesis of sucrose require the addition of catalytic amounts of this sugar to the system.

3. The equilibrium constant for phosphorolysis, expressed as

$$K = \frac{(\text{sucrose})(\text{phosphate})}{(\text{glucose-1-phosphate})(\text{fructose})}$$

is in the neighborhood of 0.05 at pH 6.6, and of 0.09 at pH 5.8 at 30°.

4. The phosphorylase appears to be specific for sucrose. Furthermore, no reaction is catalyzed between glucose-1-phosphate and either fructose-6-phosphate or fructose diphosphate.

5. Unlike a number of other sugars, glucose is strongly inhibitory to the phosphorolytic process, apparently by competing with sucrose for the enzyme. Phlorhizin was found to exert an unexplained small initial inhibition of the reaction in one direction only.

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# THE SKELETON AS A SOURCE OF ENDOGENOUS CITRIC ACID\*

BY ROBERT N. CLASS AND ARTHUR H. SMITH

(From the Department of Physiological Chemistry, Wayne University, College of Medicine, Detroit)

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During recent years, extensive investigation of the metabolic significance of citric acid has demonstrated that various dietary (Smith and Meyer (12)), hormonal (Shorr, Bernheim, and Taussky (11)), and acid-base (Östberg (5), Kuyper and Mattill (4)) factors are involved in the regulation of this metabolism and in the control of the level of urinary excretion of endogenous citric acid. Although it has been indicated that the major part of excreted citric acid is of endogenous origin (Schuck (9), Sherman, Mendel, and Smith (10)) and that the kidney may be involved in the production of citric acid (Orten and Smith (6)), there is need for more conclusive evidence bearing on the *in vivo* synthesis of this metabolite.

Evidence supporting the validity of the concept of endogenous production of citric acid has rested upon the results of balance experiments along with the absence of significant stores of citric acid in the organism. Thus, negative balances of considerable magnitude have been induced by the administration of alkali (Sherman, Mendel, and Smith (10), Schroeder and Smith (8)) and also through the administration of experimental rations rich in carbohydrate and in fat (Smith and Meyer (12)). The question of the possible existence of considerable reserve stores of citric acid, which under certain conditions might be mobilized and excreted in the urine, was raised by the recent discovery (Dickens (1)) that bone contains citric acid in concentrations many times that found in other body tissues and fluids.

In view of the discovery of higher than ordinary concentrations of citric acid in bone, and because of the question thus raised regarding the evidence for the formation of citric acid in the animal body, the present investigation was undertaken to determine whether or not there exists a relationship between the urinary excretion of endogenous citric acid and the citric acid content of bone.

## EXPERIMENTAL

Young mature male rats were given a protein-rich diet which is known to inhibit the endogenous production of citric acid (Smith and Meyer (12)).

\* The data in this paper are taken from a dissertation submitted by Robert N. Class to the Graduate School of Wayne University in partial fulfillment of the requirements for the degree of Master of Science in 1943.

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The animals, all of which weighed between 230 and 275 gm., were paired on the basis of equal body weight and citric acid production during a preliminary period. One rat in each pair received only the basal ration, whereas the other received the same amount of food but increased excretion of citric acid in the urine was stimulated. This was accomplished in one series by adding 250 mg. of sodium bicarbonate (in 8.25 per cent solution) to the vitamin adjuvants and in the second series by similar addition of sodium malate in an amount to furnish the same quantity of sodium as provided by the bicarbonate (see Orten and Smith (6)). The allowance of food was so adjusted that complete consumption was secured. Citric acid balances were determined over periods ranging from 9 to 54 days and the content of citric acid in the bones was determined at the end of each experimental period in both rats of a given pair. Thus the endogenous citric acid production as shown by the balances could be correlated with changes in citric acid content of the bone over the given period.

In the bicarbonate series, albino rats of the Sherman strain were used; in the malate series, rats of Strain 19058, Agouti X Copenhagen, Line 9955, were used. Both groups of rats were essentially the same in age and range of body weight. The animals were kept in individual metabolism cages over large glass funnels which were washed down with distilled water daily and the urine was collected in flasks containing 16 N sulfuric acid.

The basal diet was the following: lactalbumin 60.0, dextrin 15.0, Crisco 17.6, cod liver oil 5.0, and salt mixture (Hubbell, Mendel, and Wakeman (3)) 2.4 per cent.

The lactalbumin (Borden's Labco 15-42) was repeatedly extracted with distilled water for 10 days to remove a large part of the contained citric acid. This ration was supplemented daily with 1 cc. of a solution containing 80 mg. of liver extract (Lilly, No. 343) and 200 mg. of ryzamin-B (Burroughs Wellcome and Company) to provide the vitamin B complex. The basal diet provided 113.2 mg. of citric acid per 100 gm. and the vitamin adjuvants, 0.46 mg. per cc.

At the end of each experimental period, a pair of animals was killed with chloroform and the roughly dissected leg bones were put into boiling water for 5 minutes, after which the soft tissue was easily removed. After being dried at 96° for 1 week, the bones were weighed and allowed to dissolve in 20 cc. of 3:1 HCl for 1 to 2 days. The acid solution was diluted to 500 cc. with 0.5 N H<sub>2</sub>SO<sub>4</sub>, filtered, and 5 cc. aliquots used for analysis. Satisfactory recoveries were secured when citric acid was added (a) to the 3:1 HCl, (b) to the bone before solution in HCl, and (c) to the solution of bone in HCl. Citric acid was determined in food, urine, and bone by the method of Pucher, Sherman, and Vickery (7), the final estimation being made photometrically (Cenco photometer) with a color filter having a maximum transmission at 4250 Å.

In relating the citric acid content of the dried leg bones to that of the entire skeleton, a factor was computed from data on body weight, fresh skeleton weight, dried leg bone weight, and percentage of loss on drying as given by Donaldson and Conrow (2).

### *Results*

The results of the experiments on twelve rats with bicarbonate are summarized in Table I. The animals remained in good nutritive condition throughout the experimental period and gained weight slowly. All of the control rats showed a positive balance for citric acid, the values ranging from 6.49 to 9.47 mg. per day. The daily balance for the bicarbonate-fed animals averaged  $-0.10$  mg. There is no variation from this mean negative balance which can be correlated with the duration of the experiment; this indicates that the response of the animals to the alkali did not change with age nor with total amount of bicarbonate administered, within the limits of our experimental conditions. Two of the rats, Nos. 9 and 10, showed positive balances but the data on the differences of the balances of these rats from that of their respective pair mates indicate that endogenous citric acid was being formed by these animals also.

The indirect method of calculation used in estimating the citric acid content of the entire skeleton, together with the unknown factor of possible variation in initial citric acid concentration in the skeleton, forces the conclusion that little significance can be attached to small differences in citric acid content of the skeleton; only large differences in the total skeletal content of citrate accompanied by parallel changes in the concentration in the bone would indicate that citric acid was mobilized from the bone and excreted in the urine of animals showing a marked increase in citric acid output in the urine.

A comparison of the individual balances of the paired rats of the bicarbonate series with the differences in citric acid concentrations of the bone and the total amount in the skeleton indicates that there is little if any relation between endogenous citric acid and that in the bone in most cases. Indeed, Rat 7 with a negative balance of 34.0 mg. for the 27 day period had only 3 mg. less citric acid in the skeleton than its pair-fed control. Again, Rat 12, showing a negative balance of 36.0 mg. for the 54 day period, actually had a higher concentration in the bone and 14.5 mg. more citric acid in the skeleton than did its pair-fed control which showed a positive balance of 502 mg. Still more striking is the lack of correlation between the algebraic differences between citric acid balances and the difference in concentration in the bone of the experimental rat and its pair-fed control. Thus, Rats 10 and 3 had a difference in balances of 302 mg. of citric acid, but the former animal, which received the bicarbonate, had only 0.31 mg. less citrate per gm. of bone and only 16.8 mg.

less citrate in the entire skeleton than its pair-fed control. The data indicate that the excess excretion of citric acid over intake under these experimental conditions does not have its origin in the citric acid of the bone nor is a positive balance reflected in increased storage in the skeleton.

TABLE I

*Endogenous Citric Acid in Rats Fed Sodium Bicarbonate or Sodium Malate*

The experimental animals received 250 mg. of sodium bicarbonate or 264.8 mg. of disodium malate daily.

Series	Rat No	Days on diet	Experimental (E) or control (C.)	Total food consumption	Citric acid					
					Intake (a)	Excretion (b)	Balance		Concentration in bone*	Amount in total skeleton
							Total (a-b)	Per day (a-b)		
				gm.	mg	mg	mg	mg.	mg. per gm.	mg.
Sodium bicarbonate	11	9	E.	58	69.8	74.9	- 5 1	-0.57	6.75	80.3
	2	9	C.	58	69.8	11.4	+58.4	+6.49	6.82	91.3
	8	18	E.	157	186	197	-11.0	-0.61	6.35	71.1
	5	18	C.	157	186	22.7	+163	+9.05	6.79	76.7
	7	27	E.	232	275	309	-34.0	-1.26	6.21	62.1
	6	27	C.	232	275	62.6	+212	+7.85	6.57	65.3
	9	36	E.	322	381	348	+33.0	+0.92	5.76	69.1
	4	36	C.	322	381	40.1	+341	+9.47	5.05	57.0
	10	45	E.	404	458	388	+70.0	+1.56	6.55	69.9
	3	45	C.	404	458	85.6	+372	+8.27	6.86	86.7
	12	54	E.	484	573	609	-36.0	-0.67	6.14	82.0
	1	54	C.	484	573	70.8	+502	+9.30	6.03	67.5
Sodium malate	210	9	E.	59	70.9	144	-73.1	-8.12	5.99	72.2
	23	9	C.	59	70.9	11.2	+59.7	+6.63	5.65	69.0
	211	18	E.	148	176	229	-53.0	-2.94	6.41	85.3
	22	18	C.	148	176	17.1	+159	+8.83	6.03	79.4
	212	27	E.	191	229	491	-262	-9.70	5.85	73.7
	21	27	C.	191	229	31.8	+197	+7.29	6.21	74.1
	29	36	E.	254	304	762	-458	-12.7	5.72	73.0
	24	36	C.	254	304	100	+204	+5.67	5.95	71.8
	27	45	E.	281	339	1282	-943	-21.0	5.91	72.5
	26	45	C.	281	339	174	+165	+3.67	6.22	69.7
	28	54	E.	331	400	1096	-696	-12.9	5.91	68.8
	25	54	C.	331	400	76.8	+323	+5.98	6.21	70.0

\* Analysis of composite sample of tibiae, fibulae, femora, humeri, radii, and ulnae.

The data secured from the experimental animals receiving sodium malate and from their controls are also summarized in Table I. The citric acid intake of this group of rats was less than that of the bicarbonate series, because the basal food consumption was smaller. None of the animals gained weight and some lost weight. Despite this circumstance, the

results of the malate series demonstrate clearly that the mobilization of bone citrate is not a factor in the endogenous production of citric acid in the rat. At the outset, the data confirm the observations of Orten and Smith (6) that sodium malate owes its stimulation of endogenous citric acid production to something in addition to the sodium contained in the compound. The average daily balance of all the animals to which sodium malate was given was - 11.2 mg.; every rat in this series showed a negative balance. In spite of this fact, the differences between the experimental and control animals with respect to concentration of citric acid in bone and the total amount in the skeleton are less than in the bicarbonate series. For instance, Rat 27 excreted 943 mg. of citric acid in excess of the dietary intake during 45 days, whereas the quantity of citric acid in the skeleton was a little greater than that of the paired control. This rat excreted 10 times the citric acid found in its skeleton at the end of the experimental period.

A consideration of the differences in balances between paired animals again emphasizes the fact that the citrate in bone is not a significant source of citric acid under these conditions of stimulated endogenous production. Rat 27 excreted 1.108 gm. more citric acid than did its pair-fed control; the difference in concentration in the bone was 0.3 mg. per gm. and the difference in total amount in the skeleton was 2.8 mg.

The foregoing evidence regarding the lack of correlation between the citric acid balances in pair-fed animals and the citric acid content of the bone strongly suggests that endogenous citric acid arises from some type of synthetic metabolic process apart from the mobilization from the skeleton.

#### SUMMARY

On the basis of studies of the citric acid balance and of analysis of bone of pair-fed rats, it is concluded that the excess of citric acid excreted by the kidney after the administration of sodium bicarbonate and sodium malate does not originate in the skeleton but is a product of intermediary metabolism.

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# SYNTHESIS OF COENZYME AND FACTOR V IN ERYTHROCYTES, AND THE EXCRETION OF NICOTINIC ACID DERIVATIVES IN THE URINE FOLLOWING THE INGESTION OF NICOTINIC ACID AND NICOTINAMIDE

By CHARLES L. HOAGLAND, S. M. WARD, AND R. E. SHANK

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

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Recently attention was called to the fact that in the human organism nicotinic acid and nicotinamide are not equally effective in raising the content of factor V of the blood (1, 2). By means of an improved microbiological technique it was shown that, while the oral administration of nicotinic acid was followed regularly by a prompt elevation of the concentration of factor V in the blood, no change occurred in the level of this factor after ingestion of nicotinamide. Lwoff and Lwoff (3) have shown that either coenzyme I or coenzyme II will replace factor V in the metabolism of *Hemophilus influenzae*. This observation, together with subsequent reports of the relation of factor V to coenzymes I and II, has made it clear that the phosphopyridine nucleotides, or some important fraction thereof, may be regarded as factor V. For several years nicotinic acid and certain of its derivatives have been assigned only one rôle in animal metabolism; namely, that of providing essential blocks for the biological synthesis of coenzyme. In view of the general acceptance of this idea, the failure of nicotinamide to produce significant changes in the concentration of factor V in the blood is disturbing. It seemed advisable, therefore, to determine the influence of nicotinic acid and nicotinamide on the synthesis of coenzyme in the blood by another method, preferably one based on dehydrogenase activity, and to correlate changes in coenzyme concentration measured in this manner with changes in the content of factor V as determined by means of the technique in which *Hemophilus influenzae* is employed. That marked differences in the metabolism of nicotinic acid and nicotinamide exist, as suggested in an earlier report (1), is becoming abundantly clear, not only from studies of the nature of changes in the blood which follow the administration of these compounds, but from quantitative studies of the excretory products of nicotinic acid in the urine as well (4, 5). Therefore, in order to learn whether differences in the urinary output of nicotinic acid derivatives could be correlated with differential changes in the blood, following the administration of nicotinic acid and nicotinamide, quantitative studies of urinary derivatives have been carried out simultaneously, in certain instances, with studies of the factor V content of the blood.



### Materials and Methods

*Determination of Coenzyme I Activity by Rate of Oxidation of Lactate and Malate*—Owing to the difficulty encountered in expressing coenzyme I in absolute values, when determination is made by enzymatic means, only relative changes in activity have been recorded, in terms of carbon dioxide production from a given substrate. The values in all cases have been of sufficient magnitude and reproducibility to allow for correlation with changes in factor V as measured by the method in which *Hemophilus influenzae* is employed. In our hands methods for the determination of coenzyme II in blood have proved generally unsatisfactory; hence comparison between factor V content of the blood and coenzyme II activity was not attempted. Among the various methods tried for the determination of coenzyme I activity, that of Quastel and Wheatley (6), with certain modifications, appeared to give the best results. Of the various substrates used for testing coenzyme I activity, those of lactate and malate proved most satisfactory for our purposes.

*Measurement of Rate of Oxidation of Lactate and Malate*—3 cc. of whole blood were suspended in 6 cc. of isotonic NaCl in a 15 cc. graduated centrifuge tube and spun in a centrifuge for 20 minutes at 1500 R.P.M. The level of the packed cells was marked and the supernatant fluid discarded. The cells were then washed twice in saline, and when ready for use 4 volumes of distilled water were added. To the bottom of the Warburg flask were added 0.4 cc. of 0.16 M  $\text{NaHCO}_3$ , 0.6 cc. of isotonic NaCl, 0.2 cc. of 0.5 M NaCN, and 1.0 cc. of erythrocytes hemolyzed in 4 volumes of water. To the side arm were added 0.1 cc. of 0.16 M  $\text{NaHCO}_3$ , 0.2 cc. of ferricyanide solution (2.5 cc. of 10 per cent  $\text{Na}_3\text{Fe}(\text{CN})_6$  + 0.5 cc. of 0.16 M  $\text{NaHCO}_3$ ), and 0.5 cc. of sodium *l*-lactate or sodium *l*-malate. 0.5 cc. of sodium chloride was substituted for lactate and malate in the control flasks. A small stick of yellow phosphorus was placed in the center cup and the manometer and flask were equilibrated with a gas mixture consisting of 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$ . The manometers were placed in a water bath at 37° and shaken at 50 R.P.M. When temperature equilibrium had been achieved, the contents of the side arm were tilted into the reaction flask and after an interval of 5 minutes readings were taken every 10 minutes for 40 minutes.

Determinations of factor V were made on blood and urine by a method previously described in which the nitrite produced by *Hemophilus influenzae* was used as a measure of the content of factor V of added test materials (1). Determinations of trigonelline and acid-hydrolyzable derivatives of nicotinic acid in the urine were made by the method of Perlzweig, Levy, and Sarett (7).

## EXPERIMENTAL

Base-line values for the concentration of factor V, the rate of oxidation of lactate, and of malate when determined, and the urinary excretion of nicotinic acid derivatives were obtained in each case before ingestion of nicotinic acid or nicotinamide.

*Factor V Concentration and Rate of Oxidation of Lactate in Erythrocytes Following Ingestion of Nicotinic Acid*—Three persons whose dietary intake of nicotinic acid had been normal for several months were placed on an intake of 20 mg. of nicotinic acid per kilo of body weight per day, and frequent determinations made of the factor V concentration of the blood and of the rate of oxidation of lactate by the erythrocytes. Typical curves for one of these patients are given in Fig. 1. As revealed by previous studies,

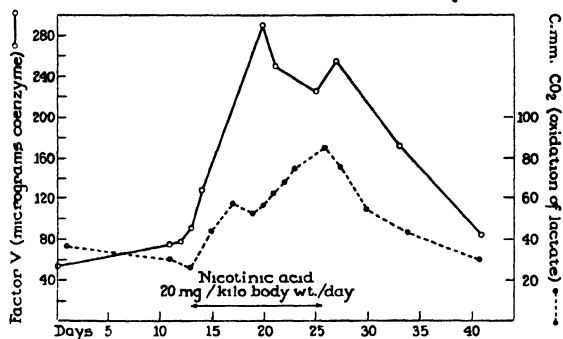


Fig. 1 Increase in the rate of oxidation of lactate and in the content of factor V following the administration of nicotinic acid.

the factor V concentration rose promptly following the ingestion of nicotinic acid. At the same time the rate of oxidation of lactate by erythrocytes was greatly enhanced. This was regarded as evidence of an increase in concentration of coenzyme I. The increased rate of oxidation of lactate is seen, moreover, to parallel the rise in factor V, and like factor V to fall slowly to the previous level when nicotinic acid was withdrawn.

With a high concentration of coenzyme in erythrocytes it might be expected that the rate of oxidation of lactate would no longer be limited by coenzyme I, but by the specific dehydrogenase of lactic acid. That this was not the case was revealed by supplying additional coenzyme I to erythrocytes already showing high coenzyme activity in order to see whether further increase in the rate of oxidation of substrate could be achieved. In every case a marked acceleration in the rate of oxidation of substrate followed the addition of more coenzyme.

**Factor V Concentration and Rate of Oxidation of Lactate and Malate Following Ingestion of Nicotinamide**—For purposes of comparing the effects obtained from nicotinic acid with those secured from nicotinamide, three additional persons were selected and given nicotinamide in amounts approximating 20 mg. per kilo of body weight per day. In this study the rate of oxidation of both lactate and malate was determined in addition to factor V. The oxidation of malate, like that of lactate, is mediated through coenzyme I. No changes were recorded in either factor V concentration in the blood or in the rate of oxidation of lactate or malate by erythrocytes, although administration of nicotinamide was continued for 20 days. However, when nicotinic acid was substituted for nicotinamide, a prompt rise occurred in factor V, followed by an increase in the rate of oxidation of both lactate and malate. These values are recorded in Fig. 2.

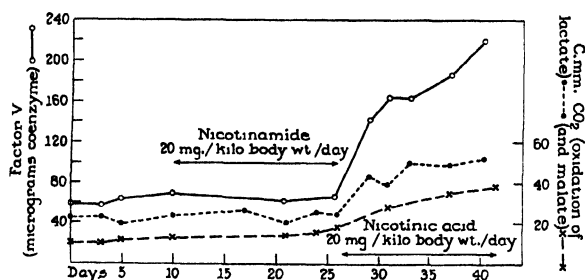


FIG. 2. The effect of the administration of nicotinamide and nicotinic acid on the concentration of factor V in the blood and on the rate of oxidation of lactate and malate by erythrocytes.

Attempts to correlate factor V with an increase in blood coenzyme have been made by Kohn and Klein, with somewhat different techniques (8). These workers claimed, however, that changes in concentration of coenzyme could not have accounted for all changes observed in factor V concentration, since factor V could increase while the rate of oxidation of lactate decreased. No results of studies with nicotinamide were reported. In no instance have we observed an increase in factor V which was accompanied by a decrease in the rate of oxidation of lactate or *vice versa*. In each instance a rise or decline in the concentration of factor V was accompanied by a parallel rise or fall in the rate of oxidation of lactate and malate. The apparent lag in the rate of oxidation of lactate, occurring during the initial rise in factor V, was attributed to a lack of sensitivity of the enzymatic method employed for the determination of coenzyme I. Relatively small increments of coenzyme are not measured by this method. The possibility that a rise in concentration of coenzyme II may be more rapid than the rise in coenzyme I, thus contributing to an accelerated rise

in total factor V, should be kept in mind. Obviously, studies on the differential rate of synthesis of coenzyme I and coenzyme II in the blood must wait until better methods for the determination of coenzyme II have been devised.

*Studies on Pattern of Excretion of Nicotinic Acid Compounds in Urine Following Ingestion of Nicotinic Acid and Nicotinamide*—Studies of the excretion of nicotinic acid derivatives in the urine following the ingestion of nicotinic acid and nicotinamide were made to learn whether the differential response of erythrocytes to these compounds was reflected by differences in the type of nicotinic acid derivatives in the urine. Three

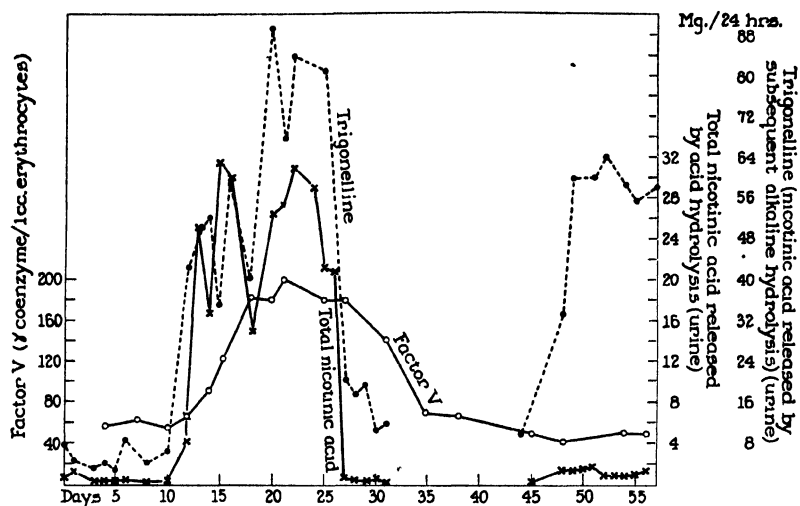


FIG. 3. The effect of the administration of nicotinic acid and nicotinamide on the concentration of factor V in the blood and on the excretion of nicotinic acid derivatives in the urine.

children, between 8 and 10 years of age, were placed on diets low in trigonelline and their urine examined quantitatively for derivatives of nicotinic acid by the method of fractionation devised by Perlzweig and associates (5). In this method total nicotinic acid is determined after acid hydrolysis, and trigonelline as nicotinic acid after subsequent hydrolysis by alkali in the presence of urea. Analyses of the blood and urine for factor V were performed at frequent intervals during the course of the experiment. When adequate base-line values for excretion had been obtained, the subjects were given nicotinic acid in amounts approximating 20 mg. per kilo of body weight per day. Administration was continued for 15 days, at which time nicotinic acid was discontinued and measurements of the excretion

of nicotinic acid compounds in the urine and of the factor V content of the blood continued until all values had returned to normal. At this time the administration of nicotinamide was begun and continued for a like period of 15 days, and determinations made as before of factor V in the blood and of the excretion of urinary compounds of nicotinic acid. A chart for one of these individuals is given in Fig. 3. Certain striking quantitative differences are immediately apparent. Following administration of nicotinic acid there was a marked and immediate rise in the urinary output of both the acid-hydrolyzable compounds of nicotinic acid and of trigonelline. A prompt rise also occurred in the content of factor V in the blood. The rise in factor V concentration, however, lagged appreciably behind the sudden increase in the rate of excretion of nicotinic acid compounds. Conversely, following cessation of the intake of nicotinic acid the urinary compounds of nicotinic acid fell abruptly to the former base-level, while the factor V content of the blood fell only slowly to its previous value. With nicotinamide, on the other hand, no important increase in the acid-hydrolyzable fraction of nicotinic acid derivatives occurred, although the output of the alkali-hydrolyzable fraction compared favorably with that observed following ingestion of nicotinic acid. Similar findings have been reported by Sarett *et al.* (5). As in previous studies, no increase in factor V in the blood occurred following the ingestion of nicotinamide, and no factor V was demonstrated in the urine following the administration of nicotinic acid or nicotinamide. In three additional subjects nicotinamide was given first, followed by nicotinic acid. No essential change in the pattern of excretion of nicotinic acid derivatives was observed, however, by varying the order of administration.

#### DISCUSSION

Since the discovery that the cell catalysts diphosphopyridine and triphosphopyridine nucleotides contain nicotinamide as an essential component of their structures, it has been tacitly assumed that nicotinic acid and its amide function primarily as building blocks for these compounds. There is increasing evidence, however, that this may not be the only rôle which nicotinic acid and nicotinamide play in the phenomena of cell catalysis. Saunders, Dorfman, and Koser (9), for example, have presented evidence which they interpret as indicating that nicotinamide, at least, is concerned in the mechanism of cellular oxidation in some way other than by the formation of the pyridine nucleotides. Results of studies described in this paper show that extraordinary differences occur between the metabolism of nicotinic acid and nicotinamide in the human organism. These differences are reflected not only in the nature of the constituents appearing in the blood, following the ingestion of nicotinic acid and nicotinamide, but

likewise in marked quantitative differences in the nicotinic acid constituents appearing in the urine. Before the whole pattern of metabolism can be fully understood, however, it will be necessary to study not only differences in the constituents of blood and urine, which appear following the administration of nicotinic acid and nicotinamide, but the nature of the compounds in tissues as well. Studies of this type are in progress and the results will be reported subsequently.

#### SUMMARY

A rise in the concentration of factor V in the blood following the administration of nicotinic acid was accompanied by a marked acceleration of the rate at which erythrocytes perform the oxidation of lactate and malate. Conversely, a fall in factor V concentration, occasioned by withdrawal of nicotinic acid, was paralleled closely by a fall in the rate of oxidation of these substrates. Nicotinamide, on the other hand, which was previously shown to be without effect on the level of factor V in the blood, produced no measurable elevation in the rate at which erythrocytes oxidize lactate and malate.

Partial fractionation of the urinary derivatives of nicotinic acid in children, following the administration of nicotinic acid, revealed a marked increase in both acid- and alkali-hydrolyzable fractions of nicotinic acid derivatives in the urine. After ingestion of nicotinamide no important change in the level of excretion of acid-hydrolyzable compounds occurred. However, a marked increase was observed in the level of excretion of trigonelline.

These results are interpreted as pointing clearly to differences in the manner in which nicotinic acid and nicotinamide are metabolized in the human organism.

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# HYDROGENASE AND NITROGEN FIXATION BY AZOTOBACTER\*

By S. B. LEE† AND P. W. WILSON

(From the Department of Agricultural Bacteriology, University of Wisconsin, Madison)

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Previous studies (1-4) have demonstrated that cultures of *Azotobacter* possess an active hydrogenase, the enzyme which catalyzes the oxidation of hydrogen. Some evidence has been uncovered which suggests that the occurrence of this enzyme is correlated with nitrogen fixation by this organism. If true, this has great significance for studies of the mechanism of biological nitrogen fixation. A detailed investigation was accordingly undertaken in which we measured the hydrogenase activity of *Azotobacter* cells grown or maintained under conditions which would vary the rate or extent of the nitrogen-fixing reaction. These included (a) the use of various forms of combined nitrogen; (b) variation in the level of combined nitrogen; (c) adaptation of cells to combined nitrogen; (d) growing cells in the presence of hydrogen and absence of free nitrogen. The general methods have been described in earlier reports; any necessary details will be furnished in the text.

*Effect of pH of Medium on Hydrogenase in Azotobacter*—In the initial experiments with combined nitrogen (2) ammonium phosphate usually served as a source of this element. During growth a pronounced decrease in the pH occurred because of selective utilization of the ammonium ion. As is shown in Table I, however, it is the presence of combined nitrogen in the medium and not the alteration of pH which is accompanied by a decrease in hydrogenase. The data demonstrate that the hydrogenase content of *Azotobacter* drops in the presence of ammonium ion, whether the pH rises, falls, or remains constant.

*Effect of Combined Nitrogen on Hydrogenase in Various Species of Azotobacter*—Cultures of *Azotobacter vinelandii*, *A. agile*, and *A. chroococcum* were grown on Burk's N-free agar medium and on the same medium plus combined nitrogen in various forms (50 mg. of N per 100 ml.). Table II gives the results of these experiments. As noted previously with *A. vinelandii* (2),  $\text{NH}_4\text{-N}$  is much more effective in reducing the hydrogenase content than is  $\text{NO}_3\text{-N}$ , whereas glutamate N has little if any effect.

Experiments by Burris (unpublished data) in this laboratory with isotopic

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† Present address, General Mills, Inc., Research Laboratories, Minneapolis.



N<sup>15</sup> have shown that fixation of free nitrogen by a culture of *Azotobacter* transferred previously on an N-free medium is not markedly inhibited by nitrate and even less by glutamate. If hydrogenase and nitrogenase are related, a marked reduction in the hydrogenase content of organisms grown

TABLE I  
Effect of pH and Nitrogen Source on Hydrogenase in *Azotobacter*

Culture grown on	N source in medium	pH after autoclaving	pH after harvest	$Q_K(N)^*$	Relative hydrogenase†
N <sub>2</sub>	N <sub>2</sub>	7.0	6.9	3980	100
	NH <sub>4</sub> NO <sub>3</sub>	7.0	6.6	1480	37.2
	NH <sub>4</sub> acetate	7.0	7.8	1920	48.2
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	N <sub>2</sub>	6.5	6.5	4170	104.8
	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> ‡	7.6	5.9	1510	38.0
	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	7.0	5.8	1530	38.2
	NH <sub>4</sub> NO <sub>3</sub>	7.0	6.4	1500	37.7
	NH <sub>4</sub> acetate	7.0	6.7	1970	49.5
	N <sub>2</sub>	7.0	6.8	3970	99.8

\*  $Q_K(N)$  is the c.mm. of total gas uptake (H<sub>2</sub>-O<sub>2</sub>) per hour per mg. of N in the cells.

† Per cent of  $Q_K(N)$  for suspension transferred and grown on N<sub>2</sub>.

‡ Nitrogen added aseptically after sterilization of the medium.

TABLE II  
Effect of Various Nitrogen Sources on Hydrogenase Content of *Azotobacter* Species

Organism	Medium	$Q_K(N)$	Relative hydrogenase*
<i>Azotobacter vinelandii</i>	N-free	4415	100
	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	1240	28.1
	KNO <sub>3</sub>	2680	60.8
	Glutamate	4395	99.5
<i>Azotobacter chroococcum</i>	N-free	2535	100
	NH <sub>4</sub> acetate	790	31.2
	KNO <sub>3</sub>	2020	81.0
	Glutamate	2589	101.8
	NH <sub>4</sub> NO <sub>3</sub>	348	13.7
<i>Azotobacter agilis</i>	N-free	3900	100
	NH <sub>4</sub> acetate	655	16.8
	KNO <sub>3</sub>	1275	32.7
	Glutamate	3290	84.5
	NH <sub>4</sub> NO <sub>3</sub>	1700	43.6

\* The hydrogenase in cells grown on an N-free medium equals 100.

in the presence of these combined nitrogen sources would therefore not be expected.

*Adaptation of Azotobacter vinelandii to Various Sources of Combined Nitrogen*—The results in Tables I and II were obtained with cultures carried in

stock on the N-free medium. Similar experiments have been made which include cultures "acclimatized" or "adapted," by previous serial transfer, to the source of nitrogen used.

*Ammonium Acetate*—50 ml. of Burk's N-free liquid medium were sterilized in Roux bottles. Varying quantities of nitrogen as ammonium acetate were then added aseptically to the flasks. One set of flasks was inoculated with a culture of *Azotobacter vinelandii* carried on the N-free medium and a similar set with a culture which had undergone six transfers on Burk's

TABLE III

*Effect of Different Concentrations of  $\text{NH}_4\text{-N}$  on Nitrogen Fixation and Hydrogenase Content of *Azotobacter vinelandii* Cultures Grown Previously on N-Free Medium and on Medium Containing  $\text{NH}_4\text{-N}$*

Culture	$\text{NH}_4$ acetate N in medium	$Q_K(\text{N})$	Relative hydrogenase	Nitrogen balance		
				Cells	Medium	Recovery*
	mg per 100 ml			total mg	mg per 100 ml	per cent
N-free	0	4740	100	10.4		
	5	4235	89.5	8.8	0.3	182.5
	10	4380	92.5	10.2	3.4	136.0
	20	3035	64.0	16.6	4.1	103.7
	30	2395	50.6	21.4	7.9	97.7
	50	1930	40.7	25.8	26.1	103.5
	70	2025	42.8	17.8	49.1	95.5
	100	2155	49.4	10.2	85.4	95.6
$\text{NH}_4$ acetate	0	4945	104	10.4		
	5	4520	95.5	7.6	0.0	152.0
	10	4980	105.0	10.4	1.2	116.0
	20	3150	66.4	17.0	3.7	103.6
	30	2475	52.2	19.4	8.1	91.7
	50	1950	41.2	27.4	19.7	94.4
	70	1245	26.2	18.8	49.7	97.8
	100	1035	21.8	14.0	83.1	97.1

\* When the recovery of N exceeds  $100 \pm 10$  per cent, the excess represents fixation of free  $\text{N}_2$ .

medium plus 500 parts per million of N as ammonium acetate. After 34 hours the cells were removed by centrifugation, washed, and the hydrogenase determined. The nitrogen in the supernatant was estimated by aeration of aliquots into standard acid after addition of NaOH.

Table III presents the data from a typical experiment. As the quantity of combined nitrogen added to the medium decreases until it is equal to or is less than the total nitrogen in the cells, increased hydrogenase in the cells is observed. This means that an increase in the nitrogen-fixing enzyme system (as indicated by a recovery of nitrogen exceeding 100 per cent) is accompanied by an increase in hydrogenase. The hydrogenase

activity of cells in the presence of lower levels of  $\text{NH}_4\text{-N}$  is the same whether the culture had been previously grown on  $\text{N}_2$  or on  $\text{NH}_4$ . It is concluded that *Azotobacter* utilizes  $\text{NH}_4\text{-N}$  at a rate that is independent of previous contact with this source of N. At higher levels (above 500 p.p.m.) the reduction in hydrogenase apparently is less with the non-adapted culture. This result is not clear cut, however, since the observed reduction in hydrogenase of the culture kept in air in this experiment was not as great as is usually noted (see, for example, Tables II and V).

*Potassium Nitrate*—In this experiment the source of nitrogen was  $\text{KNO}_3$ ; the culture was acclimatized to it by being transferred serially eight times

TABLE IV

*Effect of Different Concentrations of  $\text{KNO}_3\text{-N}$  on Nitrogen Fixation and Hydrogenase Content of *Azotobacter vinelandii* Cultures Grown Previously on N-Free Medium and on Medium Containing  $\text{KNO}_3\text{-N}$*

Culture	$\text{KNO}_3\text{-N}$ in medium <i>mg per 100 ml</i>	$Q_K(\text{N})$	Relative hydrogenase	Nitrogen balance		
				Cells	Medium	Recovery
				<i>total mg</i>	<i>mg per 100 ml.</i>	<i>per cent</i>
N-free	0	4130	100	12.2		
	5	3610	87.3	9.4	3.5	258.0
	10	3150	76.3	12.0	7.2	192.0
	25	2920	70.7	12.0	14.0	104.0
	35	2600	62.8	11.0	23.7	99.2
	50	2400	58.2	10.2	36.8	93.6
	100	2690	65.2	15.0	79.2	94.2
$\text{KNO}_3$	0	2870*	69.5*	10.0		
	5	2250	54.4	10.6	0.0	212.0
	10	1350	32.7	9.0	1.1	101.0
	25	1520	36.8	9.4	14.4	95.3
	35	1710	41.4	8.6	30.3	111.0
	50	1820	44.0	9.0	44.5	106.8
	100	1630	39.4	9.6	98.0	107.6

\* These low values may be explained by the carry-over of  $\text{NO}_3\text{-N}$  in the inoculum.

in the usual medium plus 3000 p.p.m. of  $\text{KNO}_3\text{-N}$ . The residual nitrogen in the supernatant was determined by reduction with Devarda's alloy and distillation into standard acid.

The results in Table IV show that with the non-adapted cultures  $\text{KNO}_3$ , especially at low levels, has relatively little effect on the hydrogenase content, in agreement with the fact that it does not readily compete with the nitrogen-fixing reaction (5, 6). In contrast, the hydrogenase content of the adapted culture was quite markedly reduced by low levels of  $\text{KNO}_3$ . The analytical methods were not sufficiently reliable to make it possible to determine from these data whether free nitrogen was fixed by either the adapted or non-adapted culture in the presence of excessive  $\text{KNO}_3$ .

Fig. 1 illustrates the results which are relevant for the argument that hydrogenase activity is associated with the functioning of nitrogen fixation in *Azotobacter*. Whenever  $\text{NH}_4$  is the source of combined nitrogen, the hydrogenase activity is associated with the functioning of nitrogen fixation in *Azotobacter*. Also, if  $\text{NH}_4$  is the source of combined nitrogen, the hydrogenase activity decreases with an increase in the supply of fixed nitrogen whether or not the culture has been previously adapted to  $\text{NH}_4$ . This agrees with the fact that *Azotobacter* does not require an adaptation

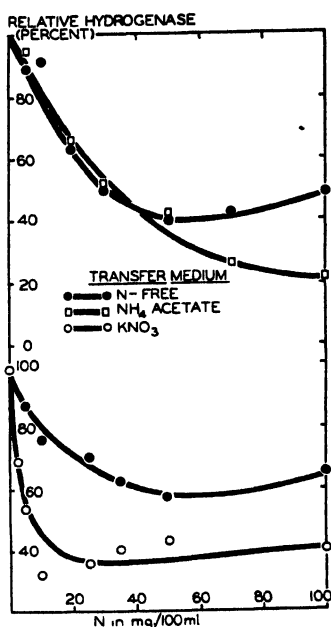


FIG. 1. Effect of different concentrations of  $\text{NH}_4$ -N and  $\text{NO}_3$ -N on nitrogen fixation and on the hydrogenase content of *Azotobacter vinelandii* cultures grown previously on an N-free medium and on a medium containing  $\text{NH}_4$ -N and  $\text{NO}_3$ -N respectively.

to  $\text{NH}_4$  before this source of nitrogen successfully competes with  $\text{N}_2$ . If  $\text{NO}_3$ -N is supplied the organism, however, the quantitative aspects of the decrease in hydrogenase depend on whether the culture has been adapted to nitrate N. With non-adapted cultures nitrogen fixation is not entirely suppressed by  $\text{NO}_3$ -N (5, 6); as can be seen in the figure, the formation of hydrogenase likewise is not markedly reduced.

*Effect of Growth in  $\text{H}_2$ - $\text{O}_2$  Gas Mixture on Hydrogenase Activity of *Azotobacter vinelandii**—The effect of the specific substrate on the hydrogenase content of *Azotobacter* was determined by growing the organisms in an  $\text{H}_2$ - $\text{O}_2$  mixture which forces the cells to obtain all nitrogen from the sub-

strate, as fixation cannot occur. There should be little, if any, nitrogen-fixing enzyme formed; consequently, if hydrogenase and nitrogenase are closely related, such cells should have less hydrogenase activity.

The technique for growing the cells is essentially that of Wyss and Wilson (7). 50 ml. of Burk's medium are placed in liter Pyrex bottles, in the center of each of which is a test-tube ( $24 \times 100$  mm.) containing 15 ml. of 20 per cent KOH. Each bottle is closed with a sterile, gas-tight rubber stopper through which is passed a gas inlet tube that terminates inside the KOH "well." A sterile cotton filter in the line also aids in preventing contamination from the incoming gas. The desired gas mixture ( $p_{H_2}$  0.8,

TABLE V  
*Effect of Growth in  $H_2$ - $O_2$  Gas Mixture on Hydrogenase Activity of  
*Azotobacter vinelandii**

Culture	Medium	Gas phase	$Q_K(N)$	Relative hydrogenase
N-free	N-free	Air	3310	100
	NH <sub>4</sub> acetate	"	990	29.9
	" "	H <sub>2</sub> -O <sub>2</sub>	623	18.8
	KNO <sub>3</sub>	Air	1160	35.0
	"	H <sub>2</sub> -O <sub>2</sub>	632	19.1
	NH <sub>4</sub> NO <sub>3</sub>	Air	900	27.2
NH <sub>4</sub> acetate	"	H <sub>2</sub> -O <sub>2</sub>	324	9.8
	N-free	Air	3070	92.8
	NH <sub>4</sub> acetate	"	1040	31.4
	" "	H <sub>2</sub> -O <sub>2</sub>	782	23.6
KNO <sub>3</sub>	N-free	Air	2540	77.0
	KNO <sub>3</sub>	"	422	12.6
	"	H <sub>2</sub> -O <sub>2</sub>	95	2.9
NH <sub>4</sub> NO <sub>3</sub>	N-free	Air	2470	74.8
	NH <sub>4</sub> NO <sub>3</sub>	"	460	13.9
	"	H <sub>2</sub> -O <sub>2</sub>	290	8.8

$p_{O_2}$  0.2 atmosphere) is added to the bottles by drawing a vacuum of 26 inches of mercury and filling it with hydrogen. This is repeated three times; then a vacuum of 5.8 to 6.0 inches is drawn and O<sub>2</sub> added to zero pressure. The bottles are connected through a water seal to a manifold which leads to a reservoir of O<sub>2</sub>. During the test CO<sub>2</sub> from respiration by the bacteria is absorbed in the KOH with a consequent pressure deficit which draws in more oxygen to replace that respired.

Table V gives data from an experiment in which two types of inoculum were used, one serially transferred on an N-free medium and a second on media containing the homologous combined nitrogen sources. All combined nitrogen was added to the bottles at the rate of 500 p.p.m., and the cells were grown for 34 hours.

In agreement with previous results, adaptation to ammonium acetate had little effect on the reduction of hydrogenase by this source of nitrogen, but adaptation to  $\text{KNO}_3$  or  $\text{NH}_4\text{NO}_3$  was followed by a greater reduction in hydrogenase when supplied with these sources of fixed nitrogen. With either adapted or non-adapted cultures, growth in the  $\text{H}_2\text{-O}_2$  gas mixture, far from increasing the hydrogenase content, actually resulted in a decrease. This was especially marked in the adapted  $\text{KNO}_3$  culture in which the hydrogenase was practically eliminated, even though the specific substrate,  $\text{H}_2$ , was present.

#### DISCUSSION

The evidence concerned with the association of hydrogenase activity and the nitrogen nutrition of *Azotobacter* provided in this report may be summarized as follows:

1. In the presence of low levels of combined nitrogen hydrogenase activity parallels, at least roughly, nitrogen fixation by *Azotobacter*. Initiation of nitrogen fixation through depletion of the combined nitrogen in the medium is accompanied by a sharp rise in the hydrogenase activity of the organisms.

2. It has been demonstrated with several species of *Azotobacter* that sources of combined nitrogen which do not readily inhibit nitrogen fixation also do not materially lower the hydrogenase content of the organism.

3. When organisms are acclimatized to sources of combined nitrogen not readily utilizable (for example,  $\text{NO}_3$ ), the nitrogen source not only inhibits nitrogen fixation more effectively, but also reduces the activity of hydrogenase.

4. The presence of the specific substrate for hydrogenase during the growth of *Azotobacter* does not cause a rise in the formation of the enzyme. Cells grown in the presence of  $\text{H}_2\text{-O}_2$  and combined nitrogen must obtain their nitrogen from the substrate; since fixation is not possible, a minimum nitrogenase activity would be expected. Coincidental with this, a minimum hydrogenase content occurs in the cells.

The major weakness of the data is the apparent lack of quantitative agreement between the activity of the nitrogen-fixing system and hydrogenase. In several of the experiments conditions were such that nitrogen fixation was entirely suppressed, but in only a few instances was hydrogenase activity completely absent. It must be realized, however, that whereas a quantitative estimation of hydrogenase is possible, only qualitative statements can be made about the nitrogen fixation system. In the presence of high concentrations of combined nitrogen, nitrogen fixation probably ceases, but whether this is due to the absence of the enzyme or to competitive inhibition by the combined nitrogen with  $\text{N}_2$  cannot be stated

with certainty. It may be that there occurs merely a reduction, not an elimination, of the nitrogenase and that this reduced content of enzyme is completely inhibited by the combined nitrogen source. The activity of the hydrogenase need not be affected by the competition, since it is known to differ from nitrogenase in many of its properties. In the complete absence of  $N_2$  we can be sure that no fixation occurs, but not so certain that this implies complete absence of the nitrogen-fixing enzyme. If the latter, it appears that nitrogenase is a highly adaptive enzyme formed only in the presence of the particular substrate. It is significant that hydrogenase likewise appears to be adaptive but responds to the presence of  $N_2$  and not  $H_2$ .

Another observation which apparently conflicts with the view that the enzyme system responsible for nitrogen fixation in *Azotobacter* may be concerned with the oxidation of  $H_2$  is the inability to demonstrate an active hydrogenase in the components of the symbiotic nitrogen-fixing system (4). This opposing evidence may not be serious, however, when it is realized that the root nodule bacteria alone are unable to fix nitrogen and that results with excised nodules are erratic (8). Hydrogenase would not be expected, therefore, to be found in pure cultures of the bacteria and would probably be weak in excised nodules. Its detection with present methods may not be feasible, especially in view of the fact that demonstration of the enzyme, even in *Azotobacter*, is sometimes difficult in the presence of organic sources of carbon. The use of heavy hydrogen may supply a more sensitive method (4). The failure of the intact symbiotic system to utilize measurable quantities of hydrogen gas might also be explained by the observation that hydrogenase frequently appears to be inhibited by the presence of organic carbon substrates.

#### SUMMARY

Sources of combined nitrogen which readily inhibit nitrogen fixation by *Azotobacter* also inhibit formation of hydrogenase. This result does not arise because of concomitant changes in pH.

Adaptation of the organism to nitrate nitrogen so that the latter is more readily assimilated causes an increase in the effectiveness of  $NO_3$  to inhibit both nitrogen fixation and hydrogenase formation.

Inhibition of nitrogen fixation by combined nitrogen in an  $H_2$ - $O_2$  atmosphere is accompanied by a marked decrease in hydrogenase, even though its specific substrate,  $H_2$ , is present. Its formation appears to respond more to the presence of  $N_2$  than to  $H_2$ .

From these results it is concluded that hydrogenase is closely related to the nitrogen-fixing system in *Azotobacter*.

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# NEW ANHYDRIDES OF PEPTIDES AND DEHYDROGENATED PEPTIDES

By JOSEPHINE E. TIETZMAN, DAVID G. DOHERTY, AND MAX BERGMANN

*(From the Laboratories of The Rockefeller Institute for Medical Research, New York)*

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The transformation of dehydrogenated peptides into anhydrides has been observed in three instances. In the first of these, acetyldehydrophenylalanyldehydrophenylalanine (I) (1) was heated with a water-pyridine mixture at 90° for 4 hours. Under these conditions, 1 molecule of water was split off, and a solution of the pyridine salt of an anhydride was formed. On the addition of HCl, the free anhydroacetyldehydrophenylalanyldehydrophenylalanine,  $C_{20}H_{18}O_3N_2$ , was obtained as yellowish crystals which melted at 210–212°. The anhydride was also formed at 37°, but very much more slowly. The unsaturated anhydride is easily soluble in aqueous bicarbonate. It forms, on treatment with diazomethane, a methyl ester, from which it is regenerated by saponification with  $N$  NaOH. This indicates that the anhydropeptide contains a carboxyl group and that it must, therefore, correspond either to Formula II or Formula IIa. The unsaturated anhydride is not appreciably altered by contact with the equivalent amount of  $N$  HCl at 28° for several days. After a solution of the anhydropeptide in  $N$  NaOH has been kept at room temperature for 20 hours, most of the anhydropeptide may be recovered on acidification.

The unsaturated anhydropeptide,  $C_{20}H_{18}O_3N_2$ , is also obtained when the azlactone (III) of acetyldehydrophenylalanyldehydrophenylalanine is heated with pyridine and water. This formation of the anhydride ring is all the more remarkable, since the heterocycle of simpler azlactones, such as the azlactone of acetyldehydrophenylalanine, is opened hydrolytically when they are heated with pyridine and water. Whether such a hydrolysis, leading from the azlactone (III) to the open chain peptide (I), occurs as an intermediary step in the transformation of (III) to (II), (IIa), has, at present, not been decided.

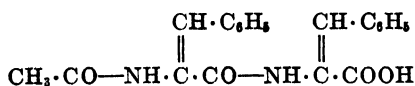
Formulas II and IIa are in good agreement with the result of the catalytic hydrogenation of the unsaturated anhydropeptide. When palladium black was used as the catalyst, the anhydropeptide took up 4 hydrogen atoms in the course of 100 to 150 hours, and two well defined, colorless substances could easily be isolated. One of these had the composition  $C_{20}H_{22}O_4N_2$ , melted at 245–246°, and proved to be identical with an inactive acetylphenylalanylphenylalanine (Isomer A) obtained by the hydrogenation

of acetyldehydrophenylalanyldehydrophenylalanine (I) and by the hydrogenation of acetyldehydrophenylalanyl-*dl*-phenylalanine.

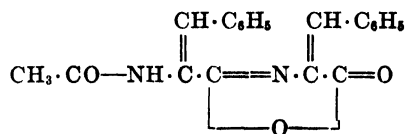
The second product obtained by the hydrogenation of the unsaturated anhydropeptide had the composition  $C_{26}H_{20}O_3N_2$ , melted at  $199-200^\circ$ , contained a carboxyl group, and formed a methyl ester melting at  $135-137^\circ$ . We regard this compound as an anhydroacetylphenylalanylphenylalanine, corresponding to Formula IV or IVa, and, consequently, as an anhydropeptide derived from saturated amino acids.

Water and  $N$  HCl at room temperature do not appreciably affect this hydrogenated anhydropeptide in the course of 24 hours. It may also be recovered after a similar interval from its solution in aqueous bicarbonate.

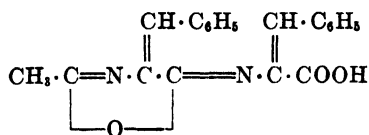
The preparation of the anhydrobenzoyldehydrophenylalanyldehydrophenylalanine, m.p.  $256-258^\circ$ , will be described in the experimental section.



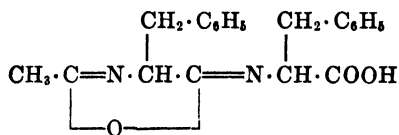
(I)



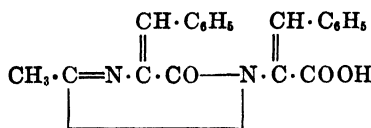
(III)



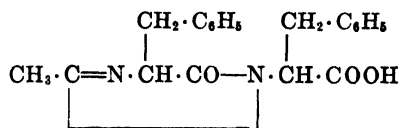
(II)



(IV)



(IIa)

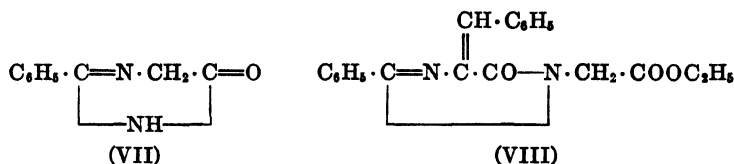
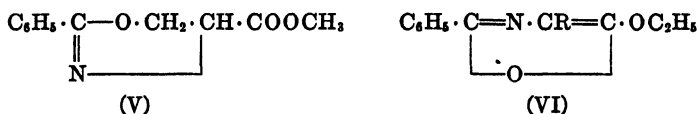


(IVa)

Heterocyclic anhydrides of structures somewhat related to that of our anhydropeptides have been obtained by the action of thionyl chloride, phosphorus pentachloride, or phosphorus pentoxide upon the esters or amides of acylated amino acids. The structures of the products thus obtained from benzoylserine methyl ester (2), from other benzoyl amino acid esters, and from benzoylglycinamide (3) are presented in Formulas V, VI, and VII. Many of these anhydrides were found to be rather sensitive towards mineral acids.

Subsequently, Graenacher and Mahler (4) demonstrated that acetyl- and benzoyldehydrophenylalanylglycine ester, when kept at  $180^\circ$  *in vacuo*,

yield anhydropeptides for which formulas such as Formula VIII were proposed. This type of reaction was not observed when the terminal glycine ester group was replaced by alanine ester or leucine ester groups. In an attempt to apply Graenacher's procedure—heating *in vacuo* at 180°—to the free acetyldehydrophenylalanylglycine, we have obtained only a dark, tarry mass. Also unsuccessful was an attempt to transform acetyl- or benzoyldehydrophenylalanylglycine into anhydropeptides by our milder pyridine-water procedure. No anhydropeptide is known as yet that has been prepared both by Graenacher's procedure and by ours. One cannot be certain, therefore, that both procedures yield products of an analogous structure.



A second reaction in which the formation of an anhydropeptide has been observed, is the well known Ploechl-Erlenmeyer azlactone synthesis. In his classic study of this reaction, Dakin (5) has shown that the azlactone formation from benzaldehyde and glycine is complicated by the formation of a polymeric benzylidene glycine. As a second by-product of the benzaldehyde-glycine condensation, a compound,  $\text{C}_{20}\text{H}_{16}\text{O}_3\text{N}_2$ , m.p. 256°, has now been isolated. It will be noted that this "by-product (256°)" has the same empirical formula as the previously discussed anhydropeptide (II), (IIa), and the azlactone (III). Any compound corresponding to a structure represented either by Formula II, IIa, or III may exist in four stereoisomers. For the azlactone (III) two stereoisomers have already been described (1).<sup>1</sup> Four compounds are thus known which have the same empirical formula. Of these, the by-product (256°) has the highest melting point. It forms well crystallized, yellow or orange, ammonium, pyridine, and sodium salts, from which it may be regenerated upon the addition of HCl. This behavior makes the presence of a carboxyl group probable. It appears possible, therefore, that either Formula II or IIa represents the structure of the by-product  $\text{C}_{20}\text{H}_{16}\text{O}_3\text{N}_2$ . The scant information at present available does not, however, permit a definite decision.

<sup>1</sup> See the foot-note on p. 627 of the paper referred to.



bath with 40 cc. of a 1:1 mixture of water and pyridine for 4 hours. A clear solution was obtained. It was acidified at 0° with about 85 cc. of 2 N HCl. An oil separated, which on the addition of ether, soon crystallized. After 24 hours at 0°, the crystals were filtered,<sup>5</sup> and twice recrystallized from 400 cc. of absolute ethanol. For maximum yield, the mixture was kept for 2 days at 0° before filtration. Yield, 8 gm. M.p., 210–212° (with decomposition).

$C_{26}H_{16}O_2N_2$ .	Calculated.	C 72.3, H 4.8, N 8.4
332.3	Found.	" 72.4, " 4.8, " 8.3

A similar experiment carried on for 20 days at 37.5° yielded 16 gm. of crude material melting at 209–210° (with decomposition). After three recrystallizations from absolute alcohol, 3.2 gm. of anhydroacetyldehydrophenylalanyldehydrophenylalanine were obtained, which melted at 210–212° (with decomposition).

Found. C 72.2, H 5.2, N 8.4

The anhydropeptide may also be prepared, at steam bath temperature, from the azlactone of acetyldehydrophenylalanyldehydrophenylalanine. The yield is, in this case, about 10 gm. of anhydropeptide from 20 gm. of azlactone. M.p., 210–212° (with decomposition).

Found. C 72.2, H 5.0, N 8.3

The unsaturated anhydropeptide forms heavy crystals of a creamy, yellow color. It is somewhat soluble in hot ethanol, much less so in cold ethanol, and very slightly soluble in water. Aqueous bicarbonate, aqueous pyridine, and solutions of sodium or ammonium acetate in warm, diluted methanol form soluble salts from which the anhydropeptide may be recovered on acidification.

*Hydrogenation of Anhydroacetyldehydrophenylalanyldehydrophenylalanine*—In contact with palladium black, a solution of 40 gm. of the unsaturated anhydropeptide in 1 liter of absolute ethanol took up 2 molecules of hydrogen. At 20–25° this took almost 150 hours. Towards the end of the hydrogenation, a colorless crystalline precipitate formed, which was collected together with the palladium black. This precipitate proved to be an acetylphenylalanylphenylalanine. Its purification will be discussed below.

The yellow alcoholic filtrate from the palladium black was evaporated *in vacuo*, twice taken up in ethyl acetate, and evaporated again. The viscous residue was taken up in another 250 cc. portion of ethyl acetate. On standing, a fine, colorless precipitate of the anhydroacetylphenylalanylphenylalanine appeared. Yield, after several days, 15 gm. It was dissolved in acetone and crystallized as heavy, rectangular prisms upon the careful addition of water. M.p., 199–200° (with decomposition). For

purification, this preparation was covered with 1 equivalent of  $N$  NaOH. After 24 hours, an equivalent amount of  $N$  HCl was added and the undissolved material twice recrystallized from acetone and water. M.p., 203–204° (with decomposition).

$C_{20}H_{20}O_2N_2$ .	Calculated.	C 71.4, H 6.0, N 8.3
336.4	Found.	" 71.1, " 6.0, " 8.1

The anhydropeptide is somewhat soluble in acetone, but very difficultly soluble in water. It is soluble in aqueous potassium bicarbonate, and may be recovered on acidification with HCl.

The methyl ester was obtained by treating the hydrogenated anhydropeptide with diazomethane. It crystallized from methanol as six-sided plates. M.p., 135–137°.

$C_{21}H_{22}O_2N_2$ .	Calculated.	C 72.0, H 6.3, N 8.0, OCH <sub>3</sub> 8.8
350.4	Found.	" 72.0, " 6.2, " 7.7, " 8.7

When the anhydropeptide was refluxed for several hours with HCl, the peptide was decomposed, and phenylalanine was formed. This amino acid was recovered as the 2,5-dibromobenzenesulfonate (6).

$C_6H_{11}O_2N \cdot C_6H_4O_2SBr_2$ .	Calculated.	C 37.4, H 3.1, N 2.9, Br 33.2
481.2	Found.	" 37.4, " 3.4, " 2.9, " 33.3

As has already been mentioned, an acetylphenylalanylphenylalanine was formed in the course of the hydrogenation and was collected with the palladium black. It was extracted with a hot 10:1 mixture of acetone and water and the filtered extract was concentrated *in vacuo*. The acetyldipeptide crystallized on the addition of water. It was recrystallized from an acetone-water mixture by the addition of more water. Rectangular plates were obtained.

$C_{20}H_{22}O_4N_2$ .	Calculated.	C 67.8, H 6.2, N 7.9
354.4	Found.	" 67.8, " 6.4, " 7.8

M.p., 245–246° (with decomposition). It will be shown later that one of the products of the hydrogenation of acetyldehydrophenylalanyldehydrophenylalanine is an acetylphenylalanylphenylalanine, melting at 246–248° (with decomposition). A mixture of the two preparations melted at 245–246°, thus indicating their identity.

*Anhydrobenzoyldehydrophenylalanyldehydrophenylalanine*—25 gm. of the azlactone of benzoyldehydrophenylalanyldehydrophenylalanine (1) were heated on the steam bath for 7 hours with a mixture of 25 cc. of pyridine and 50 cc. of water. The cooled solution was diluted with 100 cc. of water and acidified to Congo red with about 105 cc. of 3  $N$  HCl. After the addition of some ether, the anhydropeptide crystallized as heavy, yellow prisms. Yield, 20 gm. M.p., 240–245° (with decomposition). It was

twice recrystallized from absolute ethanol. M.p., 258–259° (with decomposition).

$C_{30}H_{18}O_2N_2$ .	Calculated.	C 76.1, H 4.6, N 7.1
394.4	Found.	" 76.2, " 4.6, " 7.1

The benzoylanhydropeptide is soluble in acetone, methanol, and ethanol, but very difficultly soluble in water.

*Az lactone By-Product*—200 gm. of glycine were heated with 1 liter of acetic anhydride, 330 cc. of benzaldehyde, and 120 gm. of sodium acetate (7, 5). The crude azlactone was then boiled for 2 hours with 2.5 liters of water. The dark brown residue, about 60 gm., was twice extracted at room temperature with 600 cc. of ethyl acetate and then weighed 34 to 35 gm. After two recrystallizations from absolute ethanol, 13 to 15 gm. of golden brown rods were obtained. For analysis they were dissolved in a 15:1 mixture of acetone and water, and precipitated by the addition of more water. M.p., 254–255° (with decomposition).

$C_{20}H_{16}O_2N_2$ .	Calculated.	C 72.3, H 4.8, N 8.4
332.3	Found.	" 72.2, " 4.9, " 8.3

Another sample, which was twice recrystallized from ethanol, gave almost identical figures.

The azlactone by-product is sparingly soluble in cold water and cold alcohol. It is, however, dissolved by a warm, aqueous solution of sodium acetate. The ammonium salt may be obtained as glistening, yellow platelets by adding the azlactone by-product to a solution of ammonium acetate in aqueous acetone. Most of the ammonia is given off when the dry salt is heated at 78° and 0.5 mm. over  $P_2O_5$ .

*Anhydrobis(dehydrophenylalanyl)dehydrophenylalanine Azlactone*—12.5 gm. of the azlactone of acetylbis(dehydrophenylalanyl)dehydrophenylalanine (1) were dissolved in 120 cc. of acetone and 30 cc. of N NaOH. Crystallization soon began. After 2 hours, 0.9 gm. of orange needles had separated. They were stirred for a short time with a mixture of 100 cc. of acetone, 100 cc. of water, and 8 cc. of N HCl. M.p., 288–289° (with decomposition).

$C_{27}H_{19}O_2N_3$ .	Calculated.	C 77.7, H 4.6, N 10.1
417.4	Found.	" 77.4, " 4.6, " 9.9

The azlactone anhydride, when treated with an acetone-water solution of sodium acetate, does not give a sodium salt.

*Hydrogenation of Acetyldehydrophenylalanyldehydrophenylalanine*—In this operation two stereoisomeric inactive acetylphenylalanylphenylalanines are formed. In the following description these will be called Isomer A and Isomer B.

18.4 gm. of acetyldehydrophenylalanyldehydrophenylalanine were dis-



solved in 240 cc. of water containing 8.4 gm. of  $\text{NaHCO}_3$ . After the addition of 60 cc. of colloidal palladium, this solution was hydrogenated. Slightly more than 2 molecules of hydrogen were taken up. Upon acidification with 110 cc. of  $\text{N HCl}$  a colorless precipitate formed, which occluded a good deal of catalyst. In order to remove this, the precipitate was dissolved in 500 cc. of absolute ethanol. Norit was added and the suspension was filtered through a mat of norit. On the addition of 600 cc. of water to the filtrate, 7.5 gm. of Isomer A crystallized in the form of five-sided plates. They were dissolved in a 1:1 methanol-water mixture containing  $\text{KHCO}_3$ , and reprecipitated by the addition of  $\text{HCl}$ . Melting point of Isomer A, 246–248° (with decomposition).

$\text{C}_{20}\text{H}_{22}\text{O}_4\text{N}_2$ .	Calculated.	C 67.8, H 6.2, N 7.9
354.4	Found.	" 67.8, " 6.2, " 7.8

The mother liquor of Isomer A deposited 9.3 gm. of the needle-like crystals of Isomer B, which, on recrystallization from absolute ethanol, melted at 183–185°.

Found. C 67.8, H 6.3, N 8.0

The identical products (Isomers A and B) were obtained from the hydrogenation of acetyldehydrophenylalanyl-*dl*-phenylalanine.

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# THE BIOCHEMICAL DEFECT IN NICOTINIC ACID DEFICIENCY

## II. ON THE NATURE OF THE ANEMIA

BY PHILIP HANDLER AND WILLIAM P. FEATHERSTON

(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, September 3, 1943)

Attention has frequently been called to the similarities in the anemia of pellagra and of sprue and pernicious anemia (1). Rhoads and Miller succeeded in producing a macrocytic anemia in dogs in which they had obtained a state of chronic blacktongue (2). The latter was achieved by feeding 200 gm. of raw beef daily during each of a series of repeated blacktongue attacks which were induced by a modified Goldberger diet consisting largely of black-eyed peas and casein with proper supplementation. After several such recurrent attacks the anemia was observed. This procedure was not very satisfactory, since most animals showing the signs of acute blacktongue would not take the raw beef and relatively few anemic dogs were obtained from a large series. Further, these authors were not able to establish the nature of the nutritional deficiency responsible for the anemia.

More recently it has been demonstrated (3) that the parenteral administration of physiological saline solution to dogs in acute blacktongue resulted in almost complete alleviation of the deficiency syndrome; the animals' lives were prolonged for as much as 180 days. While the concentration of blood hemoglobin appeared actually to be increased due to hemoconcentration during acute blacktongue, a profound anemia developed during the following period.<sup>1</sup> In the present work it has been demonstrated that this anemia is specifically due to nicotinic acid deficiency and evidence is presented suggesting that the anemia develops for lack of cozymase needed for the respiration of the immature erythrocytes.

### EXPERIMENTAL

#### *Methods*

The anemia under consideration was produced by several techniques. Three diets were used. Diet I was identical with the modified Goldberger diet used previously (3). Diet II consisting largely of corn-meal has been described by Koehn and Elvehjem (4). Diet III was a highly purified

<sup>1</sup> It is of interest that the protocols of Rhoads and Miller refer to the administration of saline to the dogs which they successfully made anemic by feeding suboptimal amounts of beef, although no reason for this is presented in the text.

ration, and a preliminary description of the fate of dogs on this régime has already been given (5). All animals received adequate supplementation twice weekly of thiamine, pyridoxine, and the fat-soluble vitamins; the dogs on Diet III also were given choline and calcium pantothenate.

Anorexia, infection of the oral mucosa with Vincent's organisms with consequent ropy salivation and buccal necrosis, loss of weight, bloody diarrhea, and dehydration composed the complex syndrome designated as blacktongue in this work. This syndrome appeared on Diets I and II and was successfully treated with saline. Blacktongue, without dehydration, also appeared on Diet III, but spontaneously disappeared, although no therapeutic treatment was administered. A number of animals on each diet, invariably those with poor appetites, never developed blacktongue but declined in weight from the beginning of the experimental period and gradually became anemic. However, no distinctions were found in the anemias of any of these groups and for the present purposes they may be considered as one unit.

The stools of almost all of these dogs were rendered free of parasites with tetrachloroethylene at the beginning of the experimental period and remained so through the course of the work. When the anemia had become pronounced and the dogs had lost a large bulk of their original body weight, they all appeared to have become more susceptible to infection by sarcoptic mange. Their coats became quite thin with large completely denuded areas. One or more sterile ulcers invariably appeared, particularly at the joints of the extremities.

Blood was, in each instance, drawn by external jugular venipuncture with a mixture of potassium and ammonium oxalates used as the anti-coagulant. Hemoglobin was determined as alkaline hematin by the colorimetric method of Evelyn (6). Reticulocytes were counted after being stained with brilliant cresyl blue. Hematocrits were measured in Wintrobe tubes. Plasma volumes were estimated after injection of the blue dye T-1824 by determination of the plasma concentration of the dye in the Evelyn colorimeter (7).

*Description of Anemia*—In Table I are summarized the pertinent data obtained from examination of the blood of twenty-two dogs. Each dog served as its own control. The values for the anemic dogs represent the results of the analyses of blood taken just before death in some instances, or at a satisfactorily anemic level in other animals in which successful therapy was later initiated. The animals appeared to fall into two groups. Group A consisted of those animals in which there appeared a striking increase in cell size, while those animals in which little or no change in cell size occurred were designated as Group B and are presented in this fashion in Table I.

While the anemia described in Table I was definitely macrocytic in character, the change in mean corpuscular volume was not as striking as that reported by Rhoads and Miller. However, there were no instances of microcytosis. In only one animal was a reticulocyte count above 1 per cent observed. At autopsy this dog was found to have a decidedly red, apparently hyperplastic marrow. While the mean corpuscular hemoglobin was very slightly elevated, the mean corpuscular hemoglobin concentration declined in proportion to the increase in cell size. Gastric analyses were performed on fourteen dogs and in no case was there any apparent impairment of acid secretory function. In this respect the state of the animals was unlike that of anemic pellagrins. Total van den Bergh reactions, performed on sera obtained by allowing blood to clot under oil, were consistently negative.

TABLE I  
*Hematological Findings in Chronic Nicotinic Acid Deficiency*

Group	No of dogs	State	Body weight	R b c	Hemoglobin	Hematocrit	Mean corpuscular volume	Mean corpuscular Hb	Mean corpuscular Hb concentration	Reticulocytes	Plasma proteins
			kg	millions per c.mm.	gm per 100 cc.	per cent	cu micro	micro-micro-grams	per cent	per cent	gm per 100 cc.
A	14	Normal	10.7	$6.91 \pm 0.46$	$15.2 \pm 1.2$	$45.5 \pm 6.1$	66	22	33	<1	6.5
"	14	Anemic	4.9	$2.83 \pm 0.51$	$6.8 \pm 0.9$	$25.1 \pm 4.0$	89	24	27	<1	6.1
B	14	Normal	10.2	$7.24 \pm 0.59$	$16.6 \pm 1.1$	$51.4 \pm 4.4$	71	23	32	<1	6.8
"	8	Anemic	4.7	$2.29 \pm 0.37$	$5.5 \pm 0.6$	$16.9 \pm 2.3$	74	24	32	<1	5.2

The extent of the anemia cannot be fully appreciated from the data of Table I. During the period in which the anemia developed, dogs frequently lost as much as 60 per cent of their original body weights. Further, while the hematocrit dropped from 50 to 25 per cent, there was also a marked decrease in the plasma volume. Consequently, while the hemoglobin concentration, in gm. per 100 cc., was reduced to perhaps 30 per cent of its original level, the total circulating hemoglobin decreased, in most instances, to but 15 per cent of its initial value. Table II summarizes the results obtained with seven dogs already included in Table I.

The tissues of a few dogs which died showing a severe anemia were available for histological examination. In no instance did the femur marrow appear yellow and fatty. However, while the marrow of three dogs appeared intensely red and hyperplastic, the marrow of four others appeared

quite pale. There was no apparent correlation between this difference and any of the hematological data obtained from these two groups.

Histological examination of the tissues of one dog was very kindly made by Dr. Duncan C. Hetherington of the Department of Anatomy. 1 hour before it was sacrificed the red cell count was 3.1 millions, hemoglobin 6.0 gm. per cent, hematocrit 25 per cent, reticulocytes 0.4 per cent.

"Preparations from two different portions of the spleen showed a relative absence of the usual splenic corpuscles and the sinusoidal pattern was practically obliterated by a 'reticuloendothelial' reaction. There were many macrophages filled with golden brown pigment (hemosiderin?) in the pulp of the spleen. Small lymphocytes were not present in any large quantity, whereas large cells resembling macrophages were very plentiful, as well as what may have been numerous immature lymphocytes crowding the sinusoids. When the Prussian blue reaction for demonstration of iron was performed on other sections of the spleen the golden brown pigment was replaced by an intense deposition of Prussian blue and great numbers of other cells of macrophagic type gave the iron reaction throughout the pulp of this organ.

TABLE II

*Effect of Nicotinic Acid Deficiency on Total Circulating Hemoglobin in Seven Dogs*

State	Body weight	Hematocrit	Plasma volume	Total blood volume	Hb concentration	Total Hb
	kg	per cent	cc.	cc.	gm. per 100 cc.	gm
Normal	12.2	58	712	1700	16.7	284
Anemic	5.5	24	505	664	5.9	39

"The bone marrow also contained many and much larger pigment-containing cells, which, under the stain of iron, were so blue as to appear black. Phagocytosis was marked but not as plentiful as in the spleen. The section of marrow examined for cell types gave the impression of an exhausted marrow. It was not crowded with cells; granulocytes were practically absent and there were few intermediate forms of myelocytes. Nests of erythropoietic activity were very few in number and erythropoiesis appeared to have stopped at the erythroblast level."

*Nutritional Basis for Anemia*—The appetite of dogs with blacktongue when they are treated with saline rapidly returns toward normal. However, thereafter it gradually diminishes and, during the period in which the anemia develops, such animals seldom eat more than 250 gm. of cooked food daily. To insure that the anemia was not simply the result of an inadequate protein, caloric, and iron intake, the following experiment was undertaken.

Three animals were treated with salt when in acute blacktongue. 60 days after complete remission of symptoms had occurred their blood hemoglobin concentrations had declined to 5.2, 6.7, and 8.1 gm. per cent respectively. Each animal was then given 1 cc. of 10 per cent ferric

ammonium citrate (with copper added) subcutaneously daily for 14 days. At the end of this period their hemoglobin values had declined still further to 4.8, 6.3, and 7.4 gm. per cent respectively.

Another group of three dogs was found to have hemoglobin concentrations of 6.9, 5.8, and 7.6 gm. per cent 60 days after remission of the black-tongue signs. For 10 days these animals were given 350 cc. subcutaneously daily of amigen<sup>2</sup> fortified with 10 per cent glucose. At the end of this period the blood hemoglobins were 5.0, 4.4, and 6.6 gm. per cent respectively.

At this point both forms of therapy were combined in all six dogs. For 12 days all the animals were given both iron and amigen with added glucose. Nevertheless, in each instance blood hemoglobins declined still further to 4.4, 5.1, 6.3, 4.0, 3.9, and 4.8 gm. per cent respectively. Thus it did not appear that the anemia induced in this manner was due to anorexia with consequent insufficient intake of protein, iron, or calories, but rather to a failure to utilize these dietary elements properly.

*Effect of Nicotinic Acid and of Nicotinamide*—Six dogs were used in the following experiments and the history of a typical member of the group is illustrated in Fig. 1. When the hemoglobin concentrations had declined sufficiently (6.0 gm. per cent were taken as the base-line in most cases) three dogs were given 50 mg. of nicotinamide per day for 3 days and three were given equivalent amounts of nicotinic acid. Thereafter, each dog was given 50 mg. of nicotinamide or of nicotinic acid once weekly. However, the food intake of each animal was limited immediately for the following period to an amount of food insufficient to permit any appreciable weight gain, 250 gm. per day of the experimental diet on which the anemia had been induced. In each animal there was an immediate reticulocyte response which reached a maximum of 15 to 30 per cent after 3 or 4 days and subsided about 10 days later. The red cell count, hemoglobin, and hematocrit continued to rise for 30 to 40 days.

The maximum hemoglobin concentrations obtained in this manner varied from 11.5 to 15 gm. per cent and the red cells returned to their original size in each dog. However, during this period there was no increase in body weight or plasma protein concentration. The dramatic reticulocyte response and subsequent continued hematopoietic rise in the absence of any increase in body weight or plasma protein concentration must suggest that the observed anemia is not due simply to a general state of debilitation but rather that nicotinic acid is in some way essential for normal hematopoiesis.

When the hemoglobin concentration had reached a maximum under these circumstances, the dogs' food allowance was doubled for the following 30

<sup>2</sup> A 10 per cent solution of a pancreatic digest of casein obtained from Mead Johnson and Company, Evansville, Indiana.

to 40 days. This resulted in a sharp rise in the plasma protein level but only a small increase in body weight. The animals' food allowance was then doubled again and they rapidly regained their original body weight. From these data it appears that when the cause of a metabolic disturbance which has produced a loss of body weight, plasma proteins, and hemoglobin is removed, dietary protein is utilized first to restore the normal

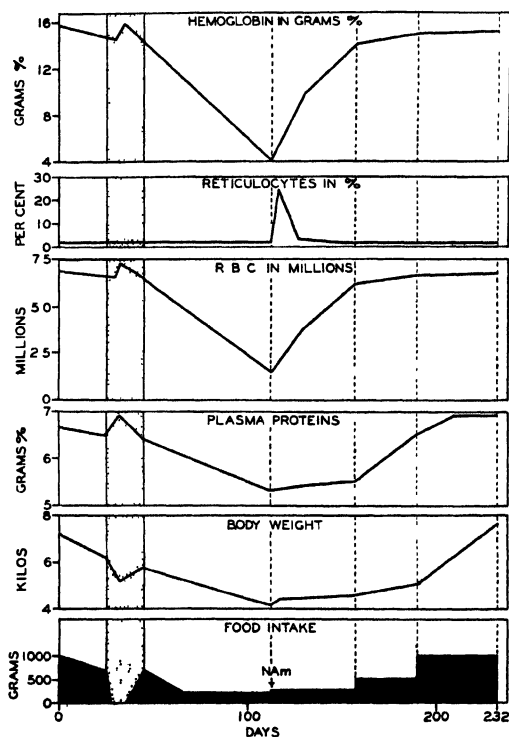


FIG. 1. The effect of nicotinamide on the anemia of chronic nicotinic acid deficiency. The stippled area represents the period in which blacktongue signs were evident and the animal was treated with saline. The arrow indicates the point at which nicotinamide was administered.

hemoglobin concentration, then plasma protein concentration, and finally to synthesize tissue proteins and restore body weight. It is noteworthy that the plasma protein concentration of only one of the total of thirty-one dogs used in this study declined sufficiently to produce a palpable edema. The abdomen of this dog filled with ascitic fluid and at autopsy the animal's heart was found to be greatly dilated.

Three of the six dogs of this group were fed Diet I, and three were fed

Diet II. Only two dogs fed Diet III were studied in this fashion. These dogs also showed the rapid hematopoietic response after nicotinamide therapy. However, while their hemoglobin concentrations rose to maxima of 10.4 and 12.3 gm. per cent 24 and 31 days after therapy was instituted, these values then dropped rather rapidly for the next week and both animals died. Such behavior, in contrast with that of the other six dogs suggests further deficiencies in this highly purified ration.

*Effects of Cobalt, Hemoglobin, and Liver Anti-Pernicious Anemia Factor*—Because of the resemblance between the anemia of pellagra and pernicious anemia in man it seemed desirable to ascertain the effect of the liver anti-pernicious anemia principle on the anemia of canine nicotinic acid deficiency. The solution used for this purpose was a concentrated liver extract containing 15 injectable units per cc.<sup>3</sup> Three nicotinic acid determinations performed by the method of Dann and Handler (8) revealed that this solution contained no more than 1.8 mg. per cc. of nicotinic acid or its amide. Five animals, all with hemoglobin concentrations below 7.5 gm. per cent, and with reticulocyte counts below 1 per cent, were each given 1 cc. of this solution intramuscularly daily for 3 days and blood samples were drawn every day for 5 days. In no instance did there appear any rise in the reticulocyte count. It appears, therefore, that the anemia of nicotinic acid deficiency does not result from any inability to produce the erythrocyte maturation factor of the liver, despite the superficial similarity between this state and pernicious anemia.

Hemoglobin, given by vein, has been found to induce a hematopoietic response in dogs rendered anemic by hemorrhage (9). It seemed of interest in the present connection to determine whether this phenomenon would occur in our animals. A solution of dog hemoglobin was prepared from washed erythrocytes obtained from normal stock dogs (9). Three dogs were each given 10 gm. of dog hemoglobin intravenously in divided doses each day for 4 days. The hemoglobin was a 10 per cent solution in 0.85 per cent NaCl. Simultaneously adequate amounts of NaHCO<sub>3</sub> and of sodium lactate were incorporated in the basal ration to prevent kidney damage from deposition on the tubules. One dog was also given similar amounts of human hemoglobin under the same conditions.<sup>4</sup> A large part of the injected dose (60 to 80 per cent) was recovered in the urine as hemoglobin and methemoglobin. No hematopoietic response as judged by reticulocyte and total erythrocyte counts was elicited by this procedure.

It has been reported (10, 11) that cobalt salts, given in excess to normal animals, induced a polycythemia. Since some evidence (12) is available

<sup>3</sup> Lederle Laboratories, Inc., Pearl River, New York.

<sup>4</sup> Very kindly provided by Dr. W. R. Amberson of the University of Maryland, Baltimore.



which suggests that the mechanism of this response may involve an inhibition of the respiration of the immature red cells, it was thought of interest to determine the effect of cobalt salts in anemia due to nicotinic acid deficiency. Three dogs were given 0.05 gm. of  $\text{Co}(\text{NO}_3)_2$  in divided doses, subcutaneously, daily for 10 days. During this period there occurred an actual diminution in the number of erythrocytes and in the hemoglobin concentration with no change in the reticulocyte count, which had been virtually nil. This is in striking contrast with the results of Mascherpa (11) and with one normal control animal in which the same quantity of cobalt elicited an increase of 2.4 gm. per cent of hemoglobin over the same period.

Because xanthopterin has recently been implicated as a possible dietary factor in erythropoiesis (13), one dog was given 45 mg. in three doses over 3 days.<sup>5</sup> The initial red cell count was 3.3 millions; the hemoglobin was 8.1 gm. per cent and the reticulocytes were less than 1 per cent. Blood samples taken on the 2nd, 4th, and 6th days after the initial dose showed no change in the reticulocyte count and the final red cell count was 3.2 millions; hemoglobin was 7.8 gm. per cent.

It should be emphasized that all of the animals in this study that failed to respond to iron, amigen, liver extract, hemoglobin, and cobalt were later successfully treated with nicotinamide or nicotinic acid. An almost equal number of dogs was studied that died during the course of one or another of the unsuccessful forms of therapy reported herein. The results obtained with these animals have not been included, since there is no *a priori* reason to believe that these animals would necessarily have responded to nicotinic acid either. This is particularly true since it has been demonstrated that there are several factors other than the anemia involved in the death of animals due to nicotinic acid deficiency in the post blacktongue state (3).

#### DISCUSSION

The anemias of pellagra are usually referred to as "being of a secondary nature" (1). This anemia in man, like that of the dogs described herein, is frequently macrocytic. However, there has been no report of the ability of nicotinic acid alone to alleviate the pellagrous anemia. Unlike the anemia of this study, the macrocytic anemia of pellagra is usually associated with gastric achlorhydria. No report of the potency of the liver anti-pernicious anemia factor, in the absence of added nicotinic acid, against the anemia of pellagra has yet appeared. Nevertheless, from the data presented herein it seems likely that the macrocytic anemia associated with a chronic state of pellagra may be specifically due to a deficiency of nicotinic acid.

The results presented above appear to establish that the anemia observed

<sup>5</sup> Very kindly provided by Dr. W. J. Darby.

in this study is specifically the result of an inadequate dietary supply of nicotinic acid. They also permit some insight into the mechanism of this hematopoietic failure. The consistently low plasma bilirubin concentrations are not compatible with excessive hemolysis as the causative factor. The decline in hemoglobin concentrations seen in dogs given supplementary iron, protein, and glucose eliminates the possibility of anemia as a reflection of a generalized undernutritional state. The negative results obtained after hemoglobin administration and the normochromic nature of the erythrocytes make it unlikely that a failure of hemoglobin synthesis is involved.

One possibility does remain which appears to be compatible with the available evidence. The only definitely established biochemical function of nicotinic acid is its synthesis to the pyridine nucleotides and subsequent rôle in cell respiration. Immature, nucleated erythrocytes respire and, presumably, must utilize the pyridine nucleotides in this respiration. Since the lifetime of the erythrocyte appears to be very short and the rate of turnover quite rapid, the requirement of nicotinic acid for the demands of erythropoiesis might be correspondingly great. Then as the supply of nicotinic acid diminishes, anemia might develop owing to lack of cozymase in the earliest stages of cell development. This situation would be aggravated by the fact that the destruction of adult red cells, with the liberation of their nicotinic acid content, occurs in the liver and spleen, so that this nicotinic acid is surrendered to the tissues at large and is not available for reutilization by the bone marrow.

The arrest of erythropoiesis at the primitive erythroblast stage observed in the histological examination of femur marrow seems quite compatible with this hypothesis. So also are the results of the experiments with cobalt. If the latter, as has been suggested (12), invokes a polycythemia by inhibiting the respiration of immature red cells at the enucleated but reticulated stage, then positive results after cobalt administration would have negated the present hypothesis. While the negative results with cobalt do not prove the hypothesis, they are at least compatible with it. Experiments designed to establish the validity of this hypothesis in a more positive fashion are now in progress.

While the present study has established the need of the erythrocytes for nicotinic acid, it is probably no less true that this is a general phenomenon. Nicotinic acid appears to be required for the growth and development of all the cells of the mammalian organism and it is the rapid rate of turnover of the erythrocytes which has permitted the particular observations of this study.

#### SUMMARY

Dogs fed three different diets deficient in nicotinic acid have been observed to develop a profound macrocytic anemia. A sharp reticulocyte

response and subsequent elevation of the red cell count and hemoglobin concentration were noted after the administration of nicotinic acid and nicotinamide. The administration of iron, protein, glucose, hemoglobin, anti-pernicious anemia factor, xanthopterin, and excessive amounts of cobalt was without any effect. A tentative mechanism for the hemato-poietic failure is discussed.

The thanks of one of us (P. H.) are due to the John and Mary R. Markle Foundation and the Duke University Research Council for their support of this work as well as to Merck and Company, Inc., of Rahway, New Jersey for a supply of the crystalline vitamins used in this work.

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# THE RAPID DETERMINATION OF ASCORBIC ACID BY THE ADAPTATION OF STOTZ'S METHOD TO PLANT MATERIALS\*

By LEONARD P. PEPKOWITZ

*(From the Department of Agricultural Chemistry, Rhode Island Agricultural Experiment Station, Kingston)*

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Recently Stotz (1) presented a rapid modification of the Bessey method for the estimation of ascorbic acid in blood and urine, dependent upon the quantitative extraction of the unreduced 2,6-dichlorophenol indophenol dye from an acid aqueous solution by xylene. Since most vegetable extracts are highly colored or turbid, this procedure offers many advantages. Most of the common colored compounds are water-soluble and do not pass into the xylene layer upon extraction; thus in most cases, correction for extraneous color in the xylene layer is avoided. In those cases in which extraneous color is taken up by the xylene, the correction is simple and better adapted for routine procedures than is the well known Bessey method (2) for the direct determination of ascorbic acid in the aqueous phase. Furthermore, only one aliquot of the given sample is necessary for the determination of ascorbic acid in the majority of materials encountered, as compared with the three aliquots required by the Bessey method or by the more recently proposed procedure of Loeffler and Ponting (3). The dye need not be standardized nor a calibration curve constructed as in the Morell procedure (4). A very important consideration is the element of time. In previous procedures galvanometer readings must be taken within 15 seconds after the addition of the dye to eliminate reactions with other reducing substances (5). In the present method, as pointed out by Stotz (1), the transfer of the unreduced dye to the xylene layer removes it from the action of these other reducing substances, eliminating the necessity for strict adherence to the time limit, since the reaction of the dye with ascorbic acid and removal to the xylene layer are accomplished within the 15 second interval. Once in the xylene layer the dye is stable for a considerable period of time, if prolonged exposure to light is avoided.

Hight and West (6) have also described a method in which the principle of the selective solubility of 2,6-dichlorophenol indophenol in xylene is utilized. In the author's hands, the Stotz procedure gives better results and is preferred for the following reasons: much greater amounts of dye

\* Contribution No. 651 of the Rhode Island Agricultural Experiment Station.

are reduced for any given amount of ascorbic acid, producing a greater deflection of the galvanometer; a simple correction for any extraneous color dissolved in the xylene, such as chlorophyll or carotenoids, can be made; the dye need not be standardized or a calibration curve be constructed. Another advantage of the proposed method is that if the amount of dye added is insufficient for the quantity of ascorbic acid contained in the aliquot more dye can be added without decreasing the accuracy of the procedure.

An analyst and an assistant can determine the ascorbic acid in 60 to 70 samples in a regular working day, including sampling and extraction.

*Reagent and Apparatus—*

The reagents and apparatus required are essentially the same as specified by Stotz (1) but will be repeated here for convenience.

A photoelectric colorimeter with a filter transmitting at 500 m $\mu$ . In the work described, a Coleman universal spectrophotometer was used.

Waring blender. It was found to be very convenient to have extra glass containers for two motor bases. This greatly increased the speed of extraction, since the extra heads could be washed and dried while others were in operation.

40 ml. heavy duty centrifuge tubes having a lip and fitted with rubber stoppers. It was found advisable to soften the stoppers by boiling them in sodium hydroxide so that they fitted the tube easily.

1.0 per cent metaphosphoric acid. This solution was made fresh every morning but if kept in a refrigerator it is satisfactory for 3 or 4 days.

2,6-Dichlorophenol indophenol. 12 mg. of the dye were dissolved in 200 ml. of warm distilled water, cooled, and filtered. The reagent is good for 10 days if stored in the refrigerator and protected from light when not in use.

0.8 N sodium hydroxide. Dissolve 32 gm. of sodium hydroxide in 1 liter of distilled water.

Phosphate-citrate buffer, pH 4.0. Mix 100 ml. of 0.1 N citric acid (1.92 gm. per 100 ml.) and 60 ml. of 0.2 N disodium phosphate (3.56 gm. per 100 ml.). It was found convenient to add the disodium phosphate to the citric acid and to adjust the pH with the aid of a Beckman pH meter.

Brom-cresol green, 0.04 per cent. Add 14.3 ml. of 0.01 N sodium hydroxide to 0.1 gm. of brom-cresol green and make to a volume of 250 ml. with distilled water.

Xylene, c.p.

*Procedure*

A weight of from 30 to 50 gm. of fresh tissue is taken as a sample. A large quantity must be used in order to give a representative sample

because of the variation in the ascorbic acid content of individual plants. The sample is homogenized in 200 ml. of 1.0 per cent metaphosphoric acid (3, 7) for 2 minutes in the Waring blender. For dehydrated vegetables 5 to 10 gm. are sufficient. If the material is extremely hard, a preliminary soaking for 15 to 30 minutes in the metaphosphoric acid is advisable.

The extract is filtered through a rapid filter paper, and the first portion of the filtrate, which will usually be cloudy, is discarded. Subsequent portions will be clear unless considerable starch is present, as in corn extracts. A portion of the filtrate containing from 0.01 to 0.10 mg. of ascorbic acid (usually 1 ml. is sufficient) is pipetted into the centrifuge tubes. This range of concentrations is not particularly critical, as excellent results have been obtained with samples as low as 0.004 mg. and as high as 0.18 mg. of ascorbic acid.

2 drops of brom-cresol green are added and 0.08 *N* sodium hydroxide is added dropwise until the indicator becomes green. Under the specified conditions, 1 or 2 drops suffice. 1 ml. of the buffer (pH 4) is then added and the tube swirled to mix the contents. Exactly 2 ml. of the dye are added from a rapid delivery pipette and again the tube is swirled. If 2 ml. are insufficient, as indicated by the complete decolorization of the dye, another 2 ml. portion of the dye can be added without affecting the accuracy of the results, but the proper correction must be made in the calculations.

10 ml. of xylene are now added with a pipette and as rapidly as possible in order to stay within the required 15 second time limit. The tube is securely stoppered and shaken moderately for 10 seconds. The complete procedure from the addition of the dye through the shaking period can be accomplished without undue haste in 15 to 20 seconds.

The tube is centrifuged for 5 minutes at a medium speed to complete the separation of the layers. After this treatment, the unreduced dye in the xylene layer will remain unchanged for several hours. The xylene is decanted into a cuvette, and with the galvanometer set at 100 with only xylene in the solvent tube, at a wave-length of 500  $m\mu$ , the deflection of the galvanometer due to the dissolved dye is noted and designated as  $G_s$ .

The control tube, which is a measure of the total dye added, is carried along with each daily run of analyses with the same dye solution. It is prepared by adding 1 ml. of buffer, 2 drops of indicator, and 2 ml. of dye to a centrifuge tube and extracting with xylene, as described above. It was found advisable to determine the control value ( $G_c$ ) in duplicate and to use the average value in order to minimize any variations due to the individual cuvettes.

However, if care is taken, the duplicate control values will vary by less than 0.2 of a galvanometer division.

The concentration of the ascorbic acid is easily calculated, for Beer's law is obeyed, as was shown by Mindlin and Butler (8) and Bessey (2).

$$C_1 = K (\log G_s - \log G_c) \quad (\text{I})$$

The value of  $C$  is a concentration factor and is usually determined as mg. of ascorbic acid per ml. of the final volume which, in this case, is 10 ml. of xylene.

The mg. of ascorbic acid in the aliquot taken is then expressed by

$$C_2 = 10K (\log G_s - \log G_c) \quad (\text{II})$$

since the dye is dissolved in 10 ml. of xylene.

Once the value for  $K$  is determined, it is constant for the same instrument and cuvettes. The value for  $K$  is ascertained by carrying known amounts of ascorbic acid through the described procedure and calculating  $K$  from Equation II. If 4 ml. of dye are used for some of the samples of the series, the value then becomes  $\log G_c = \log G_{\text{control}} - \log 2$  because of the logarithmic relationship involved as twice the depth of color is produced. This relationship holds exactly when the Coleman universal spectrophotometer is used. However, with less precise instruments, experience has shown that this relationship is less exact and that the value for  $G_c$  when twice the volume of dye is used must be determined experimentally.

Since  $C_2$  is the concentration of ascorbic acid in the aliquot taken, this value must be multiplied by the proper dilution factor to give the ml. of ascorbic acid in the sample. The quantity of water in the fresh sample cannot be neglected. Therefore, the final volume is the 200 ml. of metaphosphoric acid plus the water contained in the sample. The equation for the final calculation of the ascorbic acid content of the vegetable material as mg. per cent for an ml. aliquot is:

Mg. % ascorbic acid

$$= \frac{(C_2)(200 + \% \text{ water in sample} \times \text{weight of sample})}{\text{weight of sample}} (100) \quad (\text{III})$$

When other colored substances soluble in xylene are present in the extract, the following modification will correct for the extraneous color. Pipette a second aliquot into a centrifuge tube, and proceed as previously described, without adding any dye. This will bring the xylene-soluble pigments into the xylene layer. Two methods are available in order to correct for this extraneous color. The simplest procedure is to set the galvanometer at 100 with the described xylene extract in the solvent tube instead of pure xylene and determine  $G_s$  as before. By this means the galvanometer reading is automatically corrected for the extraneous color

present in the sample. This procedure works very well when the concentration of the extraneous color is not very great; *i.e.*, the galvanometer need not be deflected too far from the normal position to compensate for the xylene-soluble pigments.

However, if the depth of color caused by the interfering pigments is too great, a considerable loss in precision is produced by the too great displacement of the galvanometer scale. To maintain the desired precision, the galvanometer is set at 100 with pure xylene and galvanometer deflection caused by the interfering substances ( $G_s$ ) is determined.

Then

$$\text{Log } 100 - \log G_s = 2 - \log G_s = A \quad (\text{IV})$$

where  $A$  is the measure of the deflection caused by the interfering pigments. Therefore,  $\log G_s + A$  is the corrected reading and is a function of the dissolved dye alone. Accordingly, for such solutions

$$C_s = 10K (\log G_s + A - \log G_s) \quad (\text{V})$$

When a greater over-all accuracy is desired, it is advisable to make the plant extract up to a given volume (250 ml.), so that the dilution factor is known more precisely than by the use of Equation III.

#### EXPERIMENTAL

Stotz (1) has demonstrated the quantitative extraction of the non-reduced dye from acid solutions by xylene and the fact that the xylene solution of the dye follows Beer's law. This author also found agreement between Bessey's method and the xylene method for a number of blood samples.

In this laboratory, the method was further tested for its accuracy and applicability in five ways. When the same spectrophotometer and cuvettes are used, the value of  $K$  expressed as mg. of ascorbic acid per ml. should be the same, whether Bessey's method or the present method is used. This was confirmed experimentally.  $K$  determined by the present method was 0.0226, while  $K$  found by Bessey's method was 0.0233, which is excellent agreement. The recoveries of pure ascorbic acid are given in Table I. The values are the averages of four determinations.

Table II presents a series of recoveries of ascorbic acid added to a number of vegetable extracts. The values were chosen so as to cover the range of applicability of the method. The vegetable extracts listed were used because of the various coloring materials present, including both water-soluble and xylene-soluble pigments.

In order to indicate the depth of color caused by extraneous pigments in the plant extracts given in the table, the  $A$  values are also listed in Table II. These values are logarithmic as indicated in Equation IV and are



TABLE I  
*Recoveries of Pure Ascorbic Acid*

Taken	Found	
mg.	mg.	per cent
0.020	0.020	100.0
0.030	0.032	106.6
0.040	0.041	102.5
0.050	0.055	110.0
0.060	0.062	103.3
Average . . . . .		104.5

TABLE II  
*Recovery of Ascorbic Acid from Various Plant Extracts by Modified Stotz Procedure*

Extract	Ascorbic acid				
	Present	Added	Total	Found	
	mg	mg.	mg	mg.	per cent
Green pepper	0.040	0.010	0.050	0.050	100.0
		0.020	0.060	0.057	95.0
		0.020	0.060	0.058	96.7
		0.030	0.070	0.068	97.1
		0.040	0.080	0.080	100.0
" "	0.038	0.020	0.058	0.057	98.3
		0.050	0.088	0.081	92.0
		0.010	0.030	0.031	103.3
Beet root	0.020	0.020	0.040	0.037	92.5
" "	0.010	0.010	0.020	0.023	115.0
		0.020	0.030	0.032	106.7
		0.030	0.040	0.040	100.0
" top	0.003	0.020	0.023	0.024	104.3
		0.040	0.043	0.043	100.0
		0.060	0.063	0.068	107.9
		0.020	0.119	0.123	103.4
Spinach (A, 0.4389)*	0.099	0.040	0.134	0.128	95.5
" (" 0.0731)*	0.015	0.021	0.036	0.037	102.8
		0.046	0.061	0.058	95.1
		0.063	0.078	0.080	102.6
Grape juice	0.014	0.008	0.022	0.017	77.2
		0.024	0.038	0.032	84.2
		0.040	0.054	0.050	92.6
Tomato juice filtrate (A, 0.0315)*	0.011	0.011	0.022	0.022	100.0
		0.033	0.044	0.044	100.0
		0.055	0.066	0.066	100.0
Orange pulp	0.046	0.011	0.057	0.058	101.8
		0.033	0.079	0.079	100.0
		0.055	0.101	0.100	99.0
Ascorbic acid	0.013		0.013	0.013	100.0
Same + added chlorophyll (A, 0.0650)*	0.032		0.032	0.035	109.4
Average recovery . . . . .					99.1

\* The value for A is proportional to the depth of the extraneous xylene-soluble pigment.

directly proportional to the amount of xylene-soluble pigment present. The last two entries in Table II represent pure ascorbic acid solutions to which chlorophyll has been added. As indicated in Table II, the recoveries are quantitative. The average recovery for all thirty-one samples is 99.1 per cent.

A comparison of the ascorbic acid content of a series of plant extracts as determined by the Bessey method and the proposed procedure is presented in Table III. The values are mg. of ascorbic acid per ml. of extract and the results indicate the accuracy of the described procedure as compared to an established and proved method.

The fifth method of testing the described procedure consisted in determining the ascorbic acid content of standard samples. These samples had been assayed collaboratively by a variety of different methods, including

TABLE III  
*Comparison of Bessey and Proposed Methods*

Extract	Ascorbic acid per ml. extract	
	Bessey	Modified Stotz
	mg	mg.
Pepper	0.029	0.028
Celery	0.019	0.017
Cabbage	0.024*	0.018
Tomato	0.034	0.030
Grape juice	0.008	0.009

\* The higher value is probably caused by other reducing substances, as indicated by rapid drift of the galvanometer. The correction suggested by Bessey was applied but probably did not completely compensate for these other reducing substances in this particular case.

titrimetric and colorimetric procedures. For a sample of carrots, in this laboratory a value of 2.46 mg. per cent was obtained, which deviated from the mean value obtained in the other laboratories by 2.5 per cent.<sup>1</sup> A sample of corn which assayed 10.08 mg. per cent in this laboratory deviated only 0.5 per cent from the reported mean.<sup>1</sup> A third sample of peas which assayed 3.95 mg. per cent was 7 per cent higher than the average value reported.<sup>1</sup> A standard sample of tomato paste prepared by Dr. Agnes Fay Morgan<sup>2</sup> assayed 57 mg. per cent. The values reported by Dr. Morgan, which were likewise determined by a variety of methods, were 61 to 67 mg. per cent.

<sup>1</sup> Unpublished data.

<sup>2</sup> Dr. Agnes Fay Morgan, Head, Division of Home Economics, University of California.

## SUMMARY

An adaptation of Stotz's method for ascorbic acid in blood and urine is described for the rapid determination of ascorbic acid in plant materials. The method depends upon the selective solubility in xylene of non-reduced 2,6-dichlorophenol indophenol from acid solutions. Two procedures are presented to correct for extraneous xylene-soluble pigments contained in certain plant extracts. The method is applicable to all plant material tried, whether fresh, frozen, or dehydrated. Because of its simplicity, the procedure is particularly suitable for routine analyses, for 60 to 70 determinations can be made during a working day. The method is especially useful for highly colored or turbid extracts.

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# THE TURNOVER OF FATTY ACIDS IN THE CONGENITALLY OBESE MOUSE\*

BY JUAN SALCEDO, JR.,† AND DEWITT STETTEN, JR.

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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In the obese animal the chief alteration in chemical composition is the increase in the mass of depot fat. With the advent of the isotope technique it became possible to measure the rate at which the fatty acids of the body fat were being replaced, while the quantity of fat remained constant. Such studies have now been repeatedly carried out on normal mice (1-4) and it was considered of interest to compare the data so obtained with corresponding data from obese mice.

Two methods of attack are available. In the one procedure an enrichment with respect to heavy water is established and maintained in the body fluids of animals, and the rate at which deuterium is incorporated in the fatty acids of the body is studied. In the other procedure, the fats of the body are, by feeding deuterio fatty acids, initially enriched with fatty acids tagged with deuterium and the subsequent rate of decrease in isotope concentration in the fatty acids of the body is determined. As both methods give sensibly the same answer in the normal mouse at constant weight (1), we have employed the latter, more economical procedure in the study of the rate of turnover of fatty acids in the body fat of obese mice.

## EXPERIMENTAL

Four female mice from a congenitally obese strain maintained at Cold Spring Harbor were kindly placed at our disposal by Dr. E. C. MacDowell.<sup>1</sup> These animals were about 9 months old and weighed 36 to 48 gm. They were placed on a diet of dried bread-crumbs until they had achieved constant weight.

Ethyl esters of the liquid fatty acids of a sample of linseed oil were shaken

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† Fellow of the University of the Philippines, 1941-44. This report is from a thesis submitted by Juan Salcedo, Jr., in partial fulfillment of the requirements for the degree of Master of Arts in the Faculty of Pure Science, Columbia University.

<sup>1</sup> We have been informed by Dr. MacDowell that this strain of mice, known to fanciers as CHS silver, is the same as that employed by Benedict and Lee (5) in their study of the relation of body size to metabolic rate. This strain also carries a recessive gene for dwarfism.

in the presence of platinum catalyst (6) in an atmosphere of deuterium until no further uptake of gas occurred (7). The saturated deuterio fatty acid ethyl esters were purified by vacuum distillation. Deuterium,  $10.0 \pm 0.2$  atom per cent excess.

For a 5 day period the mice were offered *ad libitum* a diet of 92.5 per cent bread-crumbs and 7.5 per cent deuterio fatty acid ethyl esters. One mouse was then killed, and the survivors placed on a bread-crumb diet, and killed at 3 day intervals. During the experimental period weight gains of 1 to 2 gm. occurred. Samples of body water were obtained from each animal for deuterium analysis (8). The bodies, after removal of the gastrointestinal tracts, were treated with alcoholic KOH and the fatty acids isolated in the usual fashion (8). These fatty acids were analyzed for deuterium (9).

TABLE I

*Deuterium Content of Total Body Fatty Acids and Body Water*

Four congenitally obese mice were fed for 5 days a diet containing 7.5 per cent of deuterio fatty acid ethyl esters; D = 10.0 atom per cent excess. One animal was then killed and the survivors placed on a diet of dried bread-crumbs, and killed at intervals thereafter.

Days elapsed after fat feeding	Body weight	Total body fatty acids			Body water D
		Weight	Weight	D	
	gm.	gm	per cent body weight	atom per cent	atom per cent
0	50	19.7	39.4	0.312	0.056
3	48	19.8	41.3	0.216	0.019
6	42	16.6	39.5	0.227	0.010
9	42	17.0	40.5	0.295	0.008

## DISCUSSION

As will be seen from the data in Table I, the mice were truly obese and quite uniformly so. The percentage of fatty acids in their bodies was 39 to 41. This value is about 3 to 4 times as high as is usually observed in mice.

During the 5 day period while deuterio fatty acids were present in the diet, each mouse consumed on the average 16.4 gm. of food, containing 1.23 gm. of fatty acid ethyl ester. From a comparison of the deuterium concentrations in the material fed and in the body fatty acids of the first animal killed, it may be calculated that about 50 per cent of the labeled fatty acids consumed was deposited. The remainder may be presumed to have been burned, contributing  $D_2O$  to the body water. This distribution of labeled dietary fatty acids is perfectly normal and in excellent agreement with the results obtained by Schoenheimer and Rittenberg (8)

in parallel experiments on normal mice. The finding suggests that the obesity of our animals was not due to any tendency to deposit an abnormally large fraction of the dietary fatty acids.

In order to bring out the difference in the rates of turnover of body fatty acids in normal and obese animals, we have plotted our data, selecting as ordinates the function  $\ln(i_0/i)$ , where  $i_0$  is the isotope concentration at time  $t_0$ , and  $i$  the isotope concentration at time  $t$  thereafter. On the same coordinates we have also plotted the data obtained by Schoenheimer and Rittenberg (1) on normal mice. The apparent first order velocity constant for the reaction is, in each case, the slope of the best straight line. These lines have been calculated by the method of least squares.

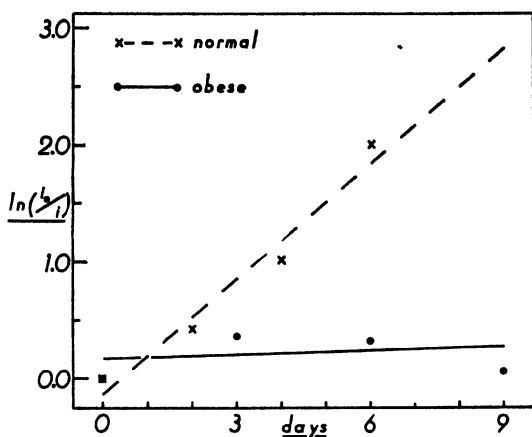


FIG. 1. The rates of disappearance of deuterium from the body fatty acids of normal and obese mice after discontinuation of feeding of deuterio fatty acid ethyl esters.

It is obvious from Fig. 1 that the rate of decrease of isotope concentration in the fatty acids of the bodies of the obese animals is much less than that of the normal mice. The half time of this reaction in normal mice on a diet of bread-crumbs has previously been estimated as 5 to 9 days (2), and more recently, on a synthetic diet, as 5 to 6 days (4). In the obese animals, the half time of this reaction is obviously much greater, though not sufficient data are at hand to make a precise estimate of its value.

From this it may be concluded that the mere presence of an excessive amount of depot fat does not serve as a stimulus to increased catabolism of fatty acids. On the contrary, the obese animals were burning rather less than normal quantities of depot fatty acids daily, despite the relatively huge mass of the depots. Because of these findings, we regard it as a

plausible assumption to attribute the obesity to a restriction of the rate of fatty acid oxidation.

• SUMMARY

Four adult congenitally obese mice were fed for 5 days a diet of bread-crumbs to which deuterio fatty acid ethyl esters were added. The animals were killed 0, 3, 6, and 9 days after the administration of isotopic fatty acids had been discontinued. Body water and total body fatty acids were analyzed for deuterium.

From a comparison of the data obtained with similar data obtained by others on normal animals, it is concluded that the proportion of dietary fatty acids directly stored in the depots of these obese mice is normal. The turnover of depot fatty acids, however, is considerably slower than in normal animals and the obesity is accordingly attributed to the retarded catabolism of the depot fatty acids.

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# SULFANILAMIDE ACETYLATION AS INFLUENCED BY VARIOUS CHEMICALS AND BY VITAMIN DEFICIENCIES

BY GUSTAV J. MARTIN AND E. H. RENNEBAUM

(From the Warner Institute for Therapeutic Research, New York)

(Received for publication, August 18, 1943)

Acetylation has long been known as a metabolic detoxication reaction (1, 2). The term, detoxication is applied incorrectly to the acetylation of sulfanilamide, as acetylsulfanilamide (3) has been demonstrated to be more toxic than the free drug. The logic of inhibiting acetylation in the clinical application of the sulfonamides has been established by reports of three types. Marshall *et al.* (3) observed increased toxicity of the acetylated form. Urolithiasis (4) is generally associated with the acetylated forms of the drugs. Finally, acetylsulfanilamide is much less effective as a chemotherapeutic agent than is the free chemical (5).

Metabolic acetylation has been studied for aromatic amines (6, 7), sulfanilamide (3), phenylaminobutyric acid (8, 9), and numerous other compounds. The problem of acetylation associated with the use of sulfonamides in clinical practice has two basic approaches: (a) that of decreasing acetylation by the elimination of chemical agents tending to increase it, *e.g.* pyruvate, and (b) that of decreasing acetylation by adding agents capable of blocking acetylation by forcing detoxication mechanisms through other channels.

## EXPERIMENTAL

Rats were maintained on the McCollum stock diet and when used weighed approximately 200 gm. All materials were given orally with one exception, in which a highly purified pyruvic acid was administered intraperitoneally. The blood samples taken at varying intervals were obtained by decapitation of the rat. In the case of vitamin-deficient rats, the dietary regimen was that presented previously (10), from which the vitamin under consideration was omitted. The rats were placed on the diet when weaned and were used for acetylation studies before food consumption was materially decreased.

By the technique of Bratton and Marshall (11) for the determination of sulfanilamide, free and combined<sup>1</sup> sulfanilamide was determined in the

<sup>1</sup> The terms "combined" or "conjugated sulfanilamide" and "acetylsulfanilamide" are used interchangeably throughout this report to indicate the increase in the values for sulfanilamide obtained after the hydrolysis in the method of Bratton and Marshall (11).



blood of rats following dosages of sulfanilamide with and without the concomitant administration of various chemicals.

Glucuronic acid, as used in this investigation and previously (12, 13), was in the form of its calcium salt and had been prepared by the hydrolysis of gum arabic. This product is impure and contains only about 6 per cent free glucuronic acid. It will be referred to as crude calcium glucuronate. Pure glucurone was prepared from ammonium menthyl glucuronate by the method of Williams (14) with rabbits. The rabbits used should be frequently changed, since the kidneys eliminate albumin which complicates the isolation. This material, which is 100 per cent glucurone (referred to throughout this communication as glucurone), was used as the standard for the determination of glucuronic acid excretion. The yield of glucurone could be increased by raising the amount of ammonium sulfate used in the precipitation of the ammonium menthyl glucuronate.

The glucuronic acid content of the various products used was determined by the use of naphthoresorcinol according to the technique of Evelyn (15).

As pointed out by Bueding,<sup>2</sup> limited accuracy is achieved in applying the naphthoresorcinol method for glucuronic acid to a hydrolysate obtained from gum arabic. Some substances (glucose, etc.) completely inhibit the development of the color; others (mucic acid, etc.) increase the color developed. The contaminating agents in the gum arabic hydrolysate would all tend to inhibit the development of the color; therefore, pending the development of improved methods it is difficult to arrive at an estimation of the glucuronic acid content of the product used.

We have noted that certain rats in any given series seem to be incapable of acetylating sulfanilamide. This fact frequently complicates the interpretation of a set of determinations. The failure of some rat livers to acetylate sulfanilamide was reported in the *in vitro* studies of Klein and Harris (16). We are now investigating the liver function of those rats which failed to conjugate sulfanilamide in our studies *in vivo*. The added uncontrollable factor of individual variation makes it necessary to use large numbers of rats in any given series. It also seems to indicate that the failure of dogs to acetylate sulfanilamide may be a positive mechanism in the sense of inhibition rather than a negative mechanism in the sense of a lack of enzyme or other factor necessary for the reaction. Furthermore, exceptional susceptibility to ordinarily non-toxic doses of sulfanilamide in rats and mice may be associated with this phenomenon.

### Results

*Sodium Acetate*—The simultaneous administration of sodium acetate with sulfanilamide reduced the degree of acetylation in the rat. 128 rats

<sup>2</sup> Bueding E., personal communication.

were used in the experiment. Each value in Table I represents an average for four sets of four rats each. Equal volumes of blood from each of four rats were pooled and an analysis run on the pooled sample. The degree of inhibition is indicated by the fact that the acetylation is reduced by 39 per cent.

*Glucuronic Acid*—Pure glucurone did not inhibit the acetylation of sulfanilamide to the extent that the crude calcium glucuronate did (13). This may be due to the fact that other agents, *e.g.* acetate, inhibit acetylation and might very well be present in the crude product. It is to be noted that in the rabbit, monkey, and human there was no corresponding inhibition of acetylation such as is produced by glucurone in the rat. 144 rats were used in this study (Table II), and the average reduction in acetylation produced by glucurone was 20 per cent.

TABLE I

*Effect of Sodium Acetate on Acetylation of Sulfanilamide (2 Gm. per Kilo) in the Rat*  
Each value equals the average for four sets of four rats each.

Sodium acetate dosage	With acetate	Control	Decreased by
gm. per kg.	per cent	per cent	per cent
4	5.07	11.08	54
8	6.87	14.88	53
1.6	6.44	13.8	49
2	5.0	10.62	53
1.6	6.6	8.13	18
2	2.7	6.74	60
2	7.36	7.79	5
2	6.76	9.28	27
Average.....	5.85	10.3	39.9

*Sodium Pyruvate*—Sodium pyruvate greatly increased the acetylation of sulfanilamide, the average value of such increase being in excess of 300 per cent. No other chemical approached this value, although acetoin was active. In Table III the results obtained with pyruvate and various other chemical agents are listed. Alanine, dihydroxyacetone, sodium oxalacetate, calcium glycerophosphate, sodium *l*-malate, glycerol, *dl*-galacturonic acid, and glycolic acid were all negative, having no effect on acetylation. Adenylic acid seemed to inhibit acetylation to a slight degree but adenosine triphosphate appeared to facilitate acetylation. Owing to the difficulty of obtaining adenosine triphosphate, it was possible to run only four rats in this experimental group, but individual analyses were made. Glycine, sodium succinate, sodium acetoacetate, ascorbic acid, and cysteine slightly facilitated acetylation. Dextrose definitely increased acetylation. Under our experimental conditions neither adrenalin nor insulin had an

effect of increasing acetylation. Quinine bisulfite was also negative in its effect.

*Vitamin Deficiency States*—Eighteen pyridoxine-deficient, thirty-five pantothenic acid-deficient, fifteen thiamine-deficient, and twenty-five riboflavin-deficient rats were used in this experiment. The control set showed an acetylation value of 9.5 per cent. In the pyridoxine- and calcium pantothenate-deficient animals acetylation was normal, showing average values of 9.2 and 9.7 per cent, respectively. The riboflavin- and thiamine-

TABLE II  
*Effect of Glucurone on Acetylation of Sulfanilamide (2 Gm. per Kilo) in Rats*

	Glucurone dosage  <i>gm per kg.</i>	Per cent of total acetylated	
		Controls, sulfanilamide	Same sulfanilamide dosage + glucurone
Rats*	2.0	4.16	5.42
	2.0	8.39	2.09
	1.7	14.2	11.5
	1.0	7.9	6.9
	1.0	9.5	5.5
	1.0	3.8	5.8
	2.0	3.4	2.8
	0.8	1.3	4.6
	1.0	3.6	0.6
Average.....		6.25	5.02
			Crude calcium glucuronate
Rabbits	2.0	52.7	56.6
Monkey	2.0	27.1	24.2
Human†	50 grains each sulfanilamide and Ca glucuronate	18.5	18.2

\* Each value equals the average for four sets of four rats each.

† Goldfarb, W., and Martin, G. J., unpublished data.

deficient rats were defective in their ability to acetylate sulfanilamide, showing values of 5.6 and 5.8 per cent respectively. The animals were used in these experiments before food consumption had been reduced in order that this factor could be eliminated.

As all blood samples throughout the study were 6 hour samples, several series were used to determine the point of attainment of maximum acetylation, and it was found that at 6 hours after treatment the degree of acetylation or per cent of the total acetylated had reached a maximum. Further,

TABLE III

*Effect of Various Other Chemicals on Acetylation of Sulfanilamide (8 Gm. per Kilo)*

Each value equals the average for four sets of four rats each.

Chemicals	Dosage	Per cent conjugation of total with chemical	Control value, sulfanilamide alone
	gm. per kg		per cent
Alanine . . . . .	2.0	4.4	3.7
" . . . . .	2.0	8.4	9.1
Glycine . . . . .	2.0	12.0	7.7
" . . . . .	4.0	10.7	2.8
Sodium pyruvate . . . . .	2.0	5.8	3.1
" " . . . . .	2.0	22.1	11.7
" " . . . . .	2.0	29.8	7.8
" " . . . . .	2.0	40.4	7.6
" " . . . . .	2.0	21.2	6.1
" " . . . . .	2.0	23.8	7.2
Dihydroxyacetone . . . . .	2.0	9.76	8.52
" . . . . .	2.0	5.13	7.66
Sodium lactate . . . . .	2.0	3.46	4.75
" " . . . . .	2.0	3.29	5.98
" " . . . . .	2.0	3.43	3.61
" oxalacetate . . . . .	1.0	4.26	4.46
" " . . . . .	1.0	2.12	2.8
Calcium glycerophosphate . . . . .	4.0	11.25	9.85
" " . . . . .	4.0	6.06	2.8
" " . . . . .	4.0	3.9	5.9
Sodium succinate . . . . .	2.0	10.8	2.8
" " . . . . .	2.0	3.5	3.3
" " . . . . .	2.0	8.4	4.8
" " . . . . .	2.0	6.5	6.6
" malate . . . . .	2.0	4.55	5.55
" " . . . . .	2.0	5.16	1.44
" " . . . . .	4.0	6.6	8.08
" " . . . . .	8.0	8.8	6.5
Glycerol . . . . .	8.0	8.34	7.7
Glycolic acid . . . . .	1.0	7.3	7.2
Sodium acetoacetate . . . . .	1.6	11.6	7.7
" " . . . . .	2.0	6.7	7.7
Dextrose . . . . .	1.6	13.8	7.7
dl-Galacturonic acid . . . . .	2.0	11.3	10.6
" " . . . . .	2.0	8.5	5.3
Ascorbic acid . . . . .	2.0	14.6	7.7
Adenylic " . . . . .	0.5	4.1	5.8
" " . . . . .	0.5	2.9	3.55
" " . . . . .	0.5	4.5	7.48
Adenosine triphosphate . . . . .	0.5	15.3	6.4
Quinine bisulfite . . . . .	0.25	3.3	7.4

TABLE III—Concluded

Chemicals	Dosage	Per cent conjugation of total with chemical	Control value, sulfanilamide alone
	<i>gm. per kg.</i>		<i>per cent</i>
Cysteine.. . . .	2.0	14.8	7.7
Acetoin .. . . .	2.0	8.6	6.2
" .. . . .	2.0	9.1	4.95
	<i>mg per kg.</i>		
Adrenalin .. . . .	2.5	6.9	7.4
" .. . . .	2.5	3.1	8.1
	<i>unit per 18 gm.</i>		
Insulin.. . . .	$\frac{1}{2}$	3.4	7.4
" .. . . .	$\frac{1}{2}$	2.9	8.1

TABLE IV

*Determination of Sulfanilamide in Blood and Urine of Rats*

All values are given in mg. per 100.0 cc. of blood. The dose of sulfanilamide = 2.0 mg. per gm. of body weight.

Group No. (2 rats in each)	Total sulfanilamide	Free sulfanilamide	Conjugated sulfanilamide	Per cent conjugation of total	Volume
Blood (6 hr. sample)					
					cc
1	19.5	18.0	1 5	7.7	
2	14.4	12 8	1.6	11.1	
3	17.2	15.1	2 1	12 2	
4	24.0	22.5	1 5	6.2	
5	14.1	13.9	0.2	1.4	
6	16.8	15 1	1.7	10.1	
7	23.3	21.9	1.4	6 0	
8	16.7	16.7	0 0	0.0	
9	20.2	19.3	0.9	4.4	
10	5.4	4.4	1.0	18.7	
Average.				7.78	
Total sulfanilamide in urine (24 hr. sample)					
1	72.7	71.5	1.2	1.65	20.0
2	41.0	41.0	0.0	0.00	13.0
3	51.5	51 0	0 5	0.97	16.0
4	112 0	110 0	2.0	1.78	37.5
5	22.8	22.4	0.4	1.75	6.5
6	32.8	32 5	0.3	0.91	10.5
7	32 8	32.8	0 0	0.00	12.0
8	12.2	11 8	0 4	3 27	8 0
9	35.6	35.4	0.2	5.60	16.5
10	14.2	12.5	1.7	12.00	6.0
Average. .. .				2.80	

as Ratish<sup>3</sup> has found no correlation between blood and urine levels of acetylation, a set was run to determine this in rats. Table IV presents the results. Table IV shows that even when rat groups are used as their own controls, there is no correlation between blood and urine values for acetylation. The values are expressed in terms of per cent of the total sulfanilamide acetylated in 6 hour blood samples and 24 hour urine samples.

#### DISCUSSION

Acetylation probably depends directly upon the formation and concentration of acetyl phosphate. Lipmann (17, 18) has suggested that all acetylation processes depend upon the formation of this compound. Our evidence supports this contention, as pyruvic acid, the oxidation of which produces the active acetyl or acetyl phosphate, increases acetylation, while acetate itself inhibits. Acetate does not provide the acetyl radical except through alteration produced in the course of metabolism. Acetylation reactions do not occur if acetate is the substrate (18). These observations check exactly our results on the acetylation of sulfanilamide. Pyruvate oxidation increases markedly the degree of acetylation; acetate oxidation decreases the degree of acetylation. No 2-carbon units tried exerted the same marked effect on acetylation as did pyruvic acid. Glycolic acid has little effect, actually of about the same order as acetate. Thus, any acetylation process such as the acetylation of sulfanilamide depends upon the system phosphopyruvic acid to pyruvic acid to acetyl phosphate. Therefore, any process decreasing the acetyl group potential should decrease the acetylation of sulfanilamide, which is for many reasons desirable in sulfonamide therapy.

Adenylic acid did not increase acetylation, which leads to the assumption that its phosphate potential was not high enough to bring about increased acetyl phosphate formation. Adenosine triphosphate did increase acetylation, which suggests that its phosphate potential was high enough to increase the formation of acetyl phosphate. These observations substantiate those of Lipmann (17, 18).

The inhibition of acetylation produced by sodium acetate was suggested by Doisy and Westerfeld (19) who stated, "sodium acetate either had no effect or decreased the acetylation [of *p*-aminobenzoic acid] in six out of seven rabbits tested." Acetate had a stimulating effect on the acetylation of sulfanilamide by liver slices (16). The amount of conjugation was also increased by pyruvate, lactate, and acetoacetate. James (20) reported a 3-fold increase in the ratio of acetylated to free sulfanilamide following the administration of acetate to mice. Several other groups (21-23) have reported that acetate is effective in increasing acetylation *in vivo*. Hensel

<sup>3</sup> Ratish, H., personal communication.

(21), working with rabbits, found a 61 per cent increase in the amount of *p*-acetylaminobenzoic acid excreted when acetate was injected.

Klein and Harris (16) noted increased acetylation of sulfanilamide *in vitro* through addition of pyruvate to tissue slices. Hensel (21) had reported a 32 per cent increase in the urinary output of *p*-acetylaminobenzoic acid following the injection of sodium pyruvate. An increase of 300 per cent in the acetylation of sulfanilamide *in vivo* in the rat followed the use of pyruvate in our experiments. There was no other chemical investigated which even closely approached the potency of pyruvate in bringing about increased acetylation.

The findings reported with a crude calcium glucuronate (13) could not be duplicated with pure glucurone. Certain preparations of crude calcium glucuronate almost completely prevented acetylation, while the pure glucurone reduced acetylation by 20 per cent. It was this fact that led us to investigate many other compounds. Our results further disclose that in rabbits, monkeys, and human beings a calcium glucuronate product which was active in rats showed no effect in inhibiting acetylation. It has been our contention that glucuronic acid fed with sulfanilamide would force the "detoxication" away from acetylation and into combination with glucuronic acid. The literature contains ample evidence of this probability. Griffith (24) reported that the feeding of glycine with benzoic acid increased the output of hippuric acid and decreased that of benzoylglucuronic acid. Csonka (25) had previously demonstrated this effect of added glycine, causing an inhibition of glucuronate formation. It is true that Scudi and Robinson (26) have reported that sulfanilamide, unlike sulfapyridine and sulfathiazole, did not stimulate glucuronic acid formation. However, our own results<sup>4</sup> have disclosed that the difference between the various sulfonamides is one of dosage as regards their effect on glucuronic acid formation. At the proper dosage sulfanilamide will cause increased glucuronic acid excretion in the rat.

The defective acetylation in thiamine-deficient rats is to be anticipated if acetyl phosphate formation is the necessary prerequisite to acetylation. In thiamine deficiency, there is a retarded oxidation of pyruvic acid, and this would automatically decrease the formation of acetyl phosphate with consequent inhibition of acetylation. It follows that the more severe the vitamin deficiency, the less the acetylation. This possibility was checked in six rats which were moribund from an acute thiamine deficiency, and in these rats the per cent of the total sulfanilamide acetylated in the blood was less than 1. This value contrasts with the value of 5.8 per cent found in thiamine deficiency before the rats become moribund and cease consuming food. The value is not conclusive but indicates a trend.

<sup>4</sup> Martin G. J., and Stenzel, W., unpublished data.

The defective acetylation of sulfanilamide noted in riboflavin-deficient rats is more difficult of explanation. The suggestion is offered that, inasmuch as the nicotinic acid-containing enzymes are substrates for the riboflavin-containing enzymes, it is highly probable that in riboflavin-deficient animals there is a defective lactic acid dehydrogenase system, the enzyme converting lactic acid to pyruvic acid. In the riboflavin-deficient rat, therefore, there is a decreased formation of pyruvic acid which accounts for the defective acetylation.

Our failure to find increased acetylation when sulfanilamide was used with quinine would seem to contradict the results of Harned and Cole (27), but actually this is not the case. Their results were on urinary sulfonamides; ours were on blood. And we have demonstrated that the two are not directly related. Further, Harned and Cole got their most striking effects after the 1st day; we report 6 hour values. The results obtained when insulin was used to increase acetylation were entirely negative. This may well be a matter of timing and detail of experimental technique, when contrasted with the positive results of Harrow *et al.* (22, 28).

Acetoin did increase acetylation as reported by Doisy and Westerfeld (19) but the degree did not begin to equal the increase produced by pyruvic acid. It is therefore suggested that acetyl phosphate is formed directly from pyruvic acid and not from acetoin or diacetyl, as suggested by Doisy and Westerfeld (19).

#### SUMMARY

The acetylation of sulfanilamide by the rat is greatly increased by pyruvate. This effect surpasses that of acetoin to such a degree as to suggest the improbability of acetoin as an intermediary between pyruvate and acetyl phosphate. Sodium acetate reduced acetylation of sulfanilamide in the rat by 39 per cent.

Acetylation of sulfanilamide was defective in rats with a thiamine or a riboflavin deficiency.

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# THE COMPONENT FATTY ACIDS OF HUMAN DEPOT FAT

BY DAVID L. CRAMER\* AND J. B. BROWN

(From the Laboratory of Physiological Chemistry, The Ohio State University, Columbus)

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The fatty acid composition of human depot fat has been the subject of few previous researches. Jaeckle (1) in 1902 reported quantitative values for palmitic, stearic, and oleic acids. Since in the methods employed other saturated and unsaturated acids now known to occur in this fat were not recognized, the results of his work must be considered as qualitative only. The first quantitative data based on the somewhat more accurate procedures of two decades ago were described by Eckstein in 1925 (2). Eckstein's results were far from complete. He found 26.6 per cent of saturated fatty acids and 63.6 per cent of unsaturated acids. By the use of certain soap procedures and partial fractional distillation of the methyl esters of the component fatty acids he reported for the first time a trace of lauric acid, about 1.00 per cent of myristic acid, and less than 1 per cent each of linoleic, linolenic, and arachidonic acids. Identification of the last three acids was made through study of the bromine addition compounds. Wagner (3) 1 year later confirmed the presence of linoleic and arachidonic acids, but not linolenic. Several more recent contributions to the chemistry of human fat have been made by Heiduschka and Handritschk (4), Cathcart and Cuthbertson (5), Cuthbertson and Tompsett (6), and Stolfi (7), but no further detailed work to evaluate the component fatty acids has appeared in spite of the obvious importance of adipose tissue in the chemistry of the human body as brought out in the interesting discussion of this subject by Wells (8).

In the present work, we have distilled the methyl esters of five specimens of human depot fat through a highly efficient electrically heated column packed with glass helices. By this procedure in each instance we had available three main fractions composed essentially of  $C_{14}$ ,  $C_{16}$ , and  $C_{18}$  esters respectively, and in addition, small intermediate and residual fractions. The main fractions from two of the specimens were then studied by crystallization procedures at low temperature, developed in this laboratory, in order to identify the esters present. Tetradecenoic and hexadecenoic acids were demonstrated in this fat for the first time. Repeated crystallization of the octadecenoic acid (oleic) present gave evidence for the presence of other octadecenoic acids than oleic. The linoleic acid in

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this fat also appears to be a mixture of isomeric octadecadienoic acids, although ordinary linoleic acid (*cis, cis*) is the principal component of this mixture. On the basis of the acids identified as a result of this work, calculations of the component fatty acids in human depot fat were made and are shown in Table I.

TABLE I  
*Per Cent of Fatty Acids in Human Fat*

Specimen No.	XIII	XII	III	XX	H-XIV
Lauric . . . . .	0.1	0.6			0.9
Myristic . . . . .	2.7	5.9	2.6	2.6	3.9
Tetradecenoic . . . . .	0.2	0.6	0.4	0.4	0.5
Palmitic . . . . .	24.0	25.0	24.7	25.4	25.7
Hexadecenoic . . . . .	5.0	6.7	7.3	5.6	7.6
Stearic . . . . .	8.4	5.8	7.7	7.7	5.2
Octadecenoic . . . . .	46.9	45.4	45.8	44.8	46.6
Octadecadienoic . . . . .	10.2	8.2	10.0	11.0	8.7
Arachidonic . . . . .	1.0	1.0	0.4	0.3	0.6
Other C <sub>20</sub> . . . . .	1.5	0.8	1.1	2.2	0.3

#### EXPERIMENTAL

*Description of Fat Specimens*—The five specimens of human fat were obtained for us at autopsies made by the Department of Pathology. The pathology in each case was not believed to be of significance in the history of the fat specimen. In other words, we consider these specimens to be reasonably normal human body fats. Autopsy records show the following: Specimen XIII, female, 53 years, cardiac hypertrophy; Specimen XII, male, 74 years, arteriosclerosis; Specimen III, male, 61 years, prostatic hypertrophy; Specimen H-XIV, male, 66 years, arteriosclerosis; Specimen XX, history unknown.

Specimens were taken mainly from the abdominal regions, pannicular, mesenteric, perirenal, etc.

*Preparation and Distillation of Methyl Esters*—The fresh adipose tissue, after being washed with cold water and cooled to  $-20^{\circ}$ , was hashed and heated on the steam bath for 2 to 5 hours. The fat was then pressed out in a lard press. Water was completely removed by warming under reduced pressure. Methyl esters were prepared in the usual manner and distilled. Care was taken that the yield of esters was practically complete, usually over 97 per cent of the weight of fat taken, so that they are representative of the fatty acids of the specimen in question. Analytical data on the original fats and their methyl esters are shown in Table II.

Specimens of the above esters were distilled through an electrically heated column, 90 cm. in length, packed with glass helices. The esters of Specimens XIII and XII were worked up in distillations of four batches and two batches, respectively, in order to have available sufficient amounts of the main fractions for intensive investigation. The procedure followed for Specimen XII was as follows: A charge of 460 gm. was distilled into six fractions and residue. The head fraction ( $C_{12}$ - $C_{14}$ ) and two small intermediates were returned to the distilling flask with a new charge of 342 gm. of esters and the mixture was distilled with special care to obtain a head fraction with any  $C_{12}$  ester present. The main fractions and residues from the two batches were combined. There were thus available from this specimen three combined main fractions, a head, and two small intermediates and residue. Final distillation and analytical data on the five specimens are presented in Table III.

TABLE II  
*Analytical Data on Specimens of Human Fats and Their Methyl Esters*

Specimen No.	Neutral fat		Methyl esters	
	I No.	Saponification No.	I No.	Mol wt.
XIII	68.9	196.5	67.8	282.3
XII	67.4	194.0	64.9	289.9
III	68.8	197.9	67.8	283.5
XX	64.8	197.3	64.4	280.4
H-XIV	68.5	195.4	69.2	285.4

Experience with this still has shown that, as a rule, the main fractions are over 95 per cent of the carbon series in question; intermediate fractions are usually small in proportion to the care taken in the distillation. These intermediate fractions have been calculated from their molecular weights as binary mixtures of the two adjacent main fractions.

The  $C_{18}$  main fractions were discontinued as soon as definite evidence of rising boiling point was observed. No attempt was made to continue the distillation up to the  $C_{20}$  main fraction which was later found to amount to only about 2 per cent of the total esters. As a result, the residual fractions were about three-fourths  $C_{18}$  esters and one-fourth  $C_{20}$  esters.

*Fractional Crystallization Studies of Nature of Main and Residual Fractions*—The main and residual fractions of Specimens XII and XIII were studied in detail by crystallization procedures at low temperature. Only the experiments on Specimen XIII are described below on account of space limitations. However, the results on the two specimens agreed very closely.

TABLE III  
Final Distillation Data on Five Specimens of Methyl Esters of Human Fat

Specimen No.	Charge	Fraction C series	Weight	Mol. wt. of esters	Mol. wt. of acids	I No.	Thiocyanogen No.	Poly-bromide No.
	gm.		gm.					
XIII	2065	12-14	16.8	239.7	225.7	11.1		
		14	12.9	242.2	228.2	6.4		
		14-16*	21.3	245.5	231.5	14.5		
		14-16	21.2	255.0	241.0	22.7		
		16	527.9	267.6	253.6	17.4		
		16-18	108.7	281.9	267.9	49.3		
		18	1167.1	296.7	282.7	87.9	75.3	
		Residual 18-20	188.7	305.5	291.5	119.6		10.0
XII	802	12-14	9.0	228.1	214.1	16.6		
		14	35.3	239.3	225.3	12.0		
		14-16	18.4	251.7	237.7	24.5		
		16	227.2	269.4	255.4	20.0		
		16-18	34.5	282.2	268.2	59.1		
		18	438.1	297.3	283.3	88.9	77.7	
		Residual 18-20	37.6	309.0	295.0	144.0		18.5
III	420	14-16	35.4	260.4	246.4	20.3		
		16	87.9	272.6	258.6	24.4		
		16-18	55.0	286.3	272.3	59.1		
		18	216.6	294.8	280.8	88.6	75.9	
		Residual 18-20	24.3	305.0	291.0	102.5		6.2
H-XIV	450	14-16	10.9	245.0	231.0	10.9		
		14-16	8.7	253.2	239.2	12.4		
		14-16	15.5	260.0	246.0	35.0		
		16	116.6	268.0	254.0	21.6		
		16-18	34.4	279.0	265.0	71.8		
		18	231.0	296.9	282.9	90.5	78.9	
		Residual 18-20	34.5	301.0	287.0	98.2		6.6
XX	635	14-16	20.0	244.0	230.0	13.7		
		16	152.5	268.5	254.5	17.4		
		16-18	52.0	279.5	265.5	55.0		
		18	350.7	297.0	283.0	91.0	77.0	
		Residual 18-20	36.0	310.0	296.0	100.5		5.3

\* This mixture of  $C_{14}$ - $C_{18}$  esters was not added to the still pot with the next batch; hence, the two  $C_{14}$ - $C_{18}$  fractions here.

#### $C_{14}$ Fraction

The  $C_{14}$  esters were crystallized by the procedure described in Chart I.

By the procedure described in Chart I there were obtained 8.5 gm. of methyl myristate which is shown to be practically pure by iodine number, molecular weight, and melting point. The combined filtrate fraction, amounting to only 1.5 gm., consists of 52 per cent of methyl tetradecenoate

and 48 per cent of methyl myristate. Since the thiocyanogen number is very close to the iodine number, the presence of acids with more than one double bond is unlikely. From the iodine number, the  $C_{14}$  main fraction is 8.1 per cent methyl tetradecenoate. On account of the small amount of material available, no attempt was made to identify the tetradecenoic acid further.

### $C_{16}$ Fraction

The crystallization procedure for the separation of the constituents of the  $C_{16}$  fraction is described in Chart II.

When the original methanol solution was cooled to  $-25^{\circ}$ , only a small amount of methyl palmitate precipitated, but when cooled further to  $-50^{\circ}$  a total of 83.0 gm. of this ester crystallized out; from the iodine number this was 97.3 per cent pure. The filtrate from this crystallization was further broken up into two fractions, the crystals of which were 94 per cent and the

CHART I  
*Crystallization of  $C_{14}$  Esters of Specimen XIII*

10 gm. $C_{14}$ esters Mol. wt. 242.2, I No. 6.4, dissolve in 400 cc. methanol, cool to $-60^{\circ}$	
$P_1$	$F_1$
Dissolve in 370 cc. methanol, cool to $-60^{\circ}$	
$P_2$	$F_2$
8.5 gm., mol. wt. 240.8, I No. 0.0, m.p. $18.4^{\circ}$	$F_1$ and $F_2$ combined, 1.5 gm., mol. wt. 239.9, I No. 57.3, thiocyanogen No. 54.8

filtrate 97.5 per cent methyl hexadecenoate. This is an almost unbelievably sharp separation and demonstrates the ease with which the crystallization procedure separates simple ester mixtures of this type. The methyl hexadecenoate was further demonstrated by reduction to methyl palmitate, and by oxidation of the acid to dihydroxypalmitic acid of known melting point. The melting point of the ester,  $-42.4^{\circ}$  to  $-41.5^{\circ}$ , is practically identical with that of a specimen of methyl hexadecenoate prepared in this laboratory from menhaden oil by Frank Smith which has been shown by disruptive oxidation to be 9,10-hexadecenoate. Since the thiocyanogen number of the ester is very close to the iodine number, it seems likely that acids more unsaturated than hexadecenoic are not present in significant amounts.

### $C_{18}$ Fraction

The  $C_{18}$  esters of Specimen XIII were assumed to consist of methyl stearate, oleate, and linoleate. The polybromide number of this fraction (0.0007) in comparison with that of the succeeding residue fraction (10.0)

CHART II  
*Fractional Crystallization of C<sub>16</sub> Methyl Esters of Specimen XIII*

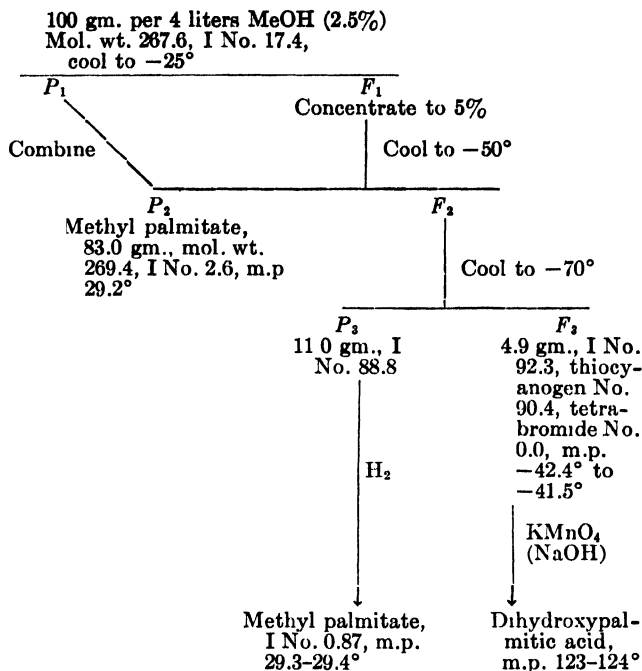
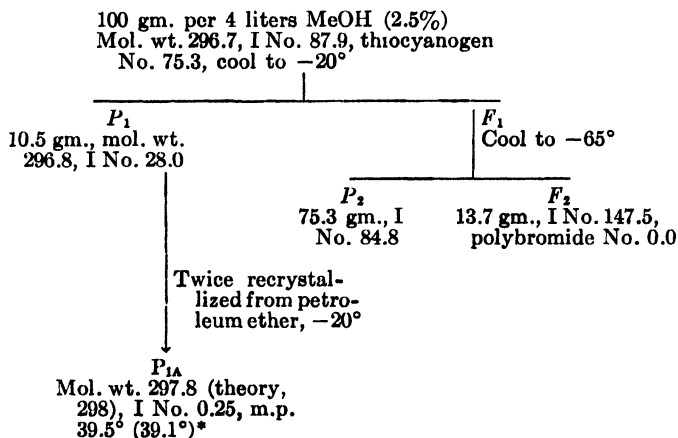


CHART III  
*Fractional Crystallization of C<sub>18</sub> Methyl Esters of Specimen XIII*



\* Reported by Francis and Piper (9).

shows that no appreciable amount of methyl arachidonate or of methyl linolenate is present in this fraction. This is also further evidence of the degree of separation attainable with the distillation apparatus used in this work.

Preliminary separation of the fraction is shown in Chart III.

The crystallization in Chart III separated the  $C_{18}$  esters into fractions composed mainly of methyl stearate ( $P_1$ ), methyl oleate ( $P_2$ ), and a mixture of oleate with linoleate ( $F_2$ ). For the further work on the methyl oleate fraction we are indebted to Dr. Carl Millican who worked up larger amounts of the  $C_{18}$  esters as part of a more general investigation in progress at the time this work was done (10). The crystallization was carried out in acetone in the earlier operations in Chart IV, because filtration is easier from this solvent than from methyl alcohol. For full details, see Chart IV.

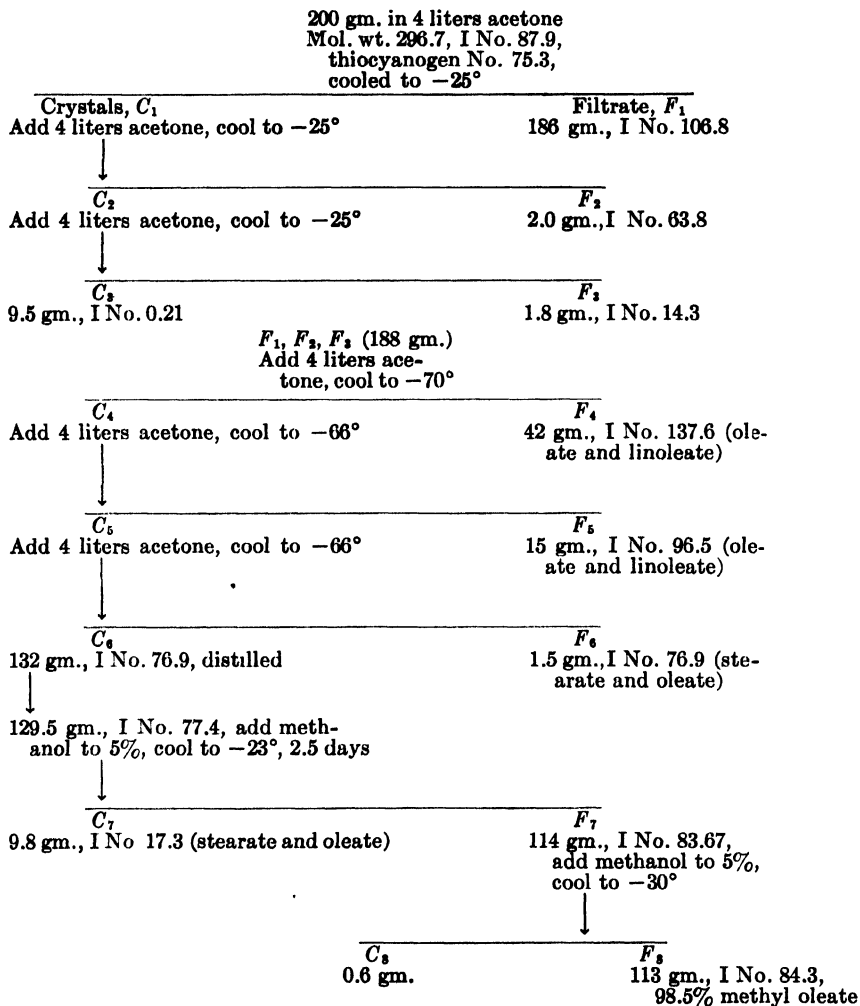
By the above procedure 113 gm. of methyl oleate of 98.5 per cent purity were isolated from 200 gm. of  $C_{18}$  esters, or about 80 per cent of the oleate in the  $C_{18}$  esters used. Oleic acid, prepared from the ester, gave the following constants: iodine number 88.8, thiocyanogen number 87.8, m.p. 12.4–12.7°. This oleic acid was recrystallized six times from petroleum ether at –60°, in order to remove any contaminating linoleic acid. The six times crystallized oleic acid gave the following results on analysis: iodine number 89.0, thiocyanogen number 87.2, mol. wt. 282.2, m.p. 12.8–13.0°. Pure oleic acid from olive oil melts at 13.3°. Both values for thiocyanogen number above are regarded by us with suspicion, because it is inconceivable that as much as 2 per cent of linoleic acid is present, as is indicated by calculation from the iodine-thiocyanogen number equation. The six consecutive filtrate fractions, amounting to 0.8 to 3.0 gm., melted at 1°, 2°, 8°, 9°, 10°, and 11°. Several of these were combined, crystallized once, and the resulting crystal fraction oxidized with alkaline permanganate by the procedure of Lapworth and Mottram (11). The resulting dihydroxystearic acid melted at 122–123°. The mixed melting point with an authentic specimen of dihydroxystearic acid prepared in a similar manner was 125–126°.

We interpret these results as follows: The octadecenoic acid of human depot fat is principally ordinary oleic acid. There are present with this oleic acid appreciable amounts of isomeric octadecenoic acids. These account for the decidedly low melting point observed in the original specimen and the 0.3–0.5° low melting point even after recrystallization. The contaminating isomers may consist, in part, of vaccenic acid (12) which has been identified in several animal fats in small amounts, or of one or more other isomers in the double bond position. The filtrate fractions in the recrystallization of the oleic acid give mixed dihydroxy derivatives of oleic and the isomeric acids. These isomers are concentrated in the filtrate frac-



tions which, as a result, have considerably lower melting points. This gradual removal of the isomers by recrystallization accounts for the rise in melting points of the several filtrates and for the appreciable rise in melting

CHART IV  
*Crystallization of  $C_{18}$  Esters of Human Fat*



point of the oleic acid in the final crystal fraction. Since the postulated isomeric octadecenoic acids have not been actually isolated in a sufficiently pure state for identification, this interpretation of data is still open to

question. Millican, in applying these crystallization procedures to several seed oils, had little difficulty in preparing oleic acid of the same melting point as the purest specimen from olive oil. However, similar preparations from a number of animal sources almost invariably resembled the oleic acid preparation from human fat.

Several of the filtrate fractions from the crystallization of the  $C_{18}$  esters which from their high iodine number were assumed to be concentrates of methyl linoleate were converted to the free acids and crystallized according to the procedure described in Chart V.

If the original fatty acid mixture used in Chart V had been composed of ordinary oleic and linoleic acids, we should have been successful in effecting a much higher concentration of the linoleic acid. Frankel, Stoneburner, and Brown (13) by the use of crystallization procedures were able to isolate

CHART V  
*Fractional Crystallization of Linoleic Acid Concentrates of Human Fat*

32.5 gm. in 425 cc. acetone Mol. wt. 281.2, I No. 125.0, cooled to $-50^{\circ}$	
$P_1$ 1.2 gm.	$F_1$ Cool to $-70^{\circ}$
$P_2$ 14.8 gm., I No. 122.3, tetrabromide No. 29.1, thiocyanogen No. 93.0	$F_2$ 16.2 gm., I No. 143.6 12.5 " in 200 cc. acetone, cooled to $-70^{\circ}$
$P_3$ 5.7 gm., I No. 145.7 5.6 " in 400 cc. petroleum ether, cooled to $-60^{\circ}$	$F_3$ 6.9 gm., I No. 139.8
$P_4$	$F_4$ 5.3 gm., I No. 146.0, tetrabromide No. 53.3, m.p. $-14.5^{\circ}$ , m.p. bromides $113.5-114^{\circ}$

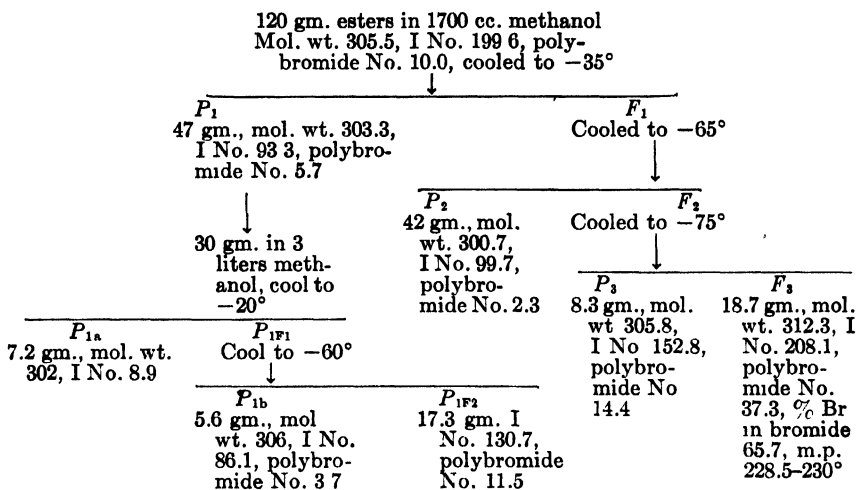
linoleic acid by crystallization of the fatty acids of a number of semidrying oils from acetone and petroleum ether. With olive oil, however, difficulty was encountered and the results were interpreted as due to the presence of mixtures of isomeric octadecadienoic acids.

$F_4$  calculated from the iodine number as a mixture of oleic and octadecadienoic acids contains 61.5 per cent of the latter (iodine number 181.0). On the basis of the tetrabromide number 102.9 the content of linoleic acid in this fraction is 51.8 per cent. The difference between these values, 9.7 per cent, is octadecadienoic acid which does not yield the usual petroleum ether-insoluble tetrabromides. In other words, about five-sixths of the octadecadienoic acid of this fraction is linoleic acid; a similar calculation for  $P_2$  shows a ratio of the two types of acids of about four-fifths.

Quantitative calculation of the composition of the  $C_{18}$  fraction can be made from iodine number-thiocyanogen number equations. These

equations are based on iodine numbers of 85.6 and 172.4 for methyl oleate and linoleate respectively, and on thiocyanogen numbers of 85.6 and 91.4 for these esters. The thiocyanogen number 91.4 for methyl linoleate was the value obtained on a specimen of pure ester prepared especially for the purpose; the determination was made by the procedure of Matthews, Brode, and Brown (14) at 16°. The equations and results given in per cent are as follows: methyl oleate =  $2.488 \times \text{thiocyanogen number} - 1.317 \times \text{iodine number} = 71.57$ ; methyl linoleate =  $1.235 (\text{iodine number} - \text{thiocyanogen number}) = 15.56$ ; methyl stearate =  $100 - (\text{oleate} + \text{linoleate}) = 12.87$ .

CHART VI  
*Fractional Crystallization of Residual Fractions of Specimen XIII*



These values have been expressed in Table I as stearic, oleic, and linoleic acids. The failure to obtain more complete separation of the unsaturated esters of this fraction is due to the probable complexity of their nature, whereby clear cut separations do not result.

#### *C<sub>20</sub> Fraction*

The total residual fraction of Specimen XIII amounted to 188.0 gm. which, from the molecular weight, consists of 138 gm. of C<sub>18</sub> esters and 50 gm. of C<sub>20</sub> esters (2.5 per cent of the original specimen). From the polybromide number of this fraction (10.0) the arachidonate content is calculated to be 1.0 per cent, leaving 1.5 per cent of other C<sub>20</sub> esters. The esters of the residual fraction were crystallized according to the procedure in Chart VI.

It is clear from the data in Chart VI that satisfactory separations were not obtained. The principal objective of the fractional crystallization, the

isolation of a concentrate of methyl arachidonate, was fairly successful in that final filtrate fraction  $F_3$ , amounting to 18.7 gm., contained 40 per cent of this highly unsaturated ester. This is calculated from the corrected factor, 91.8, of Mowry, Brode, and Brown (15). The identity of the methyl arachidonate is based on the bromine content of the polybromide, 65.7 per cent (theory for methyl arachidonate, 66.78 per cent), and its melting point, both of which values are comparable with those previously obtained by one of us (16) on similar products from a number of glandular tissues. It is likely that much better separations of  $C_{20}$  esters would be obtained if a pure  $C_{20}$  ester fraction could be prepared. However, we hesitated to try to separate these residual fractions by distillation through an electrically heated and packed column because of previous experience in this laboratory in the separation of methyl arachidonate by this method (15). The further identification of methyl arachidonate was therefore not possible. Also, it was impossible to separate and identify other possible  $C_{20}$  acids. However, we have calculated indirectly that portion of the iodine number of the  $C_{20}$  fraction which is not due to methyl arachidonate and found it to be approximately 85. This we believe to be due to the presence of an acid or acids of lesser unsaturation than arachidonic, possibly eicosenoic. It seems likely, therefore, that both this acid and arachidic acid are minor components of this fat.

#### DISCUSSION

For the first time, crystallization methods at low temperature have been applied to the study of the component fatty acids of two specimens of human body fat. Practically pure specimens of methyl myristate, palmitate, stearate, and oleate have been isolated from the esters of this fat, thus confirming previous work in this field. In addition, the presence of tetradecenoic and hexadecenoic acids in this fat has been demonstrated for the first time, these results being in line with several comparatively recent investigations on animal body fats. The oleic and linoleic acids have been shown to occur along with other isomers of these acids. Our results on the  $C_{12}$  and  $C_{20}$  fractions are incomplete. It will be necessary to work up considerably larger amounts of fat to investigate these fractions further, and positively identify the acids present with the exception of arachidonic acid, the presence of which was confirmed from analysis of its octabromide. Our results furthermore do not prove the absence of higher acids than  $C_{20}$  although these must be present, if at all, in only traces.

The demonstration of the presence in human fat of isomeric octadecenoic and octadecadienoic acids is of special interest. The synthesis of oleic acid in the animal body has been proved by numerous investigations in the past. It seems likely that the fatty acid synthesis by the animal organism results largely in the formation of ordinary oleic acid. This may be the case in

the human body. If Millican's results on the octadecenoic acids of animal origin are not in error, the isomeric acids in human fat may originate from synthesis and from the food. The finding of acids isomeric with linoleic, which is an essential fatty acid at least for rats, is worthy of special note. The most likely explanation of its occurrence is that it has been derived from similar acids in the dietary fat.

#### SUMMARY

1. The methyl esters of the fatty acids from two specimens of human depot fat were separated by distillation through an efficient column into six or seven relatively simple fractions; the main fractions representing esters of single carbon series were studied by crystallization procedures at low temperature.

2. Methyl myristate, palmitate, stearate, and oleate were isolated and identified as practically pure compounds.

3. The presence of tetradecenoic and hexadecenoic acids was demonstrated in this fat for the first time.

4. The oleic and linoleic acids of human fat are the principal  $C_{18}$  unsaturated acids present, but they are found along with isomeric octadecenoic and octadecadienoic acids.

5. The presence of arachidonic acid is confirmed.

6. From the data obtained from crystallization studies on two specimens and from distillation data on three more, the fatty acid compositions of five specimens of human fat have been calculated and recorded.

7. In the five specimens studied the linoleic (total octadecadienoic) acid contents ranged from 8.2 to 11.0 per cent; the values for arachidonic acid fell between 0.3 and 1.0 per cent.

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# A NEW METHOD FOR THE BIOASSAY OF ANTISCORBUTIC SUBSTANCES\*

ASSAYS OF DEHYDROASCORBIC ACID, 2-KETOGULONIC ACID, IRON  
ASCORBATE, AND THE EFFECTIVENESS OF ORAL AND PARENTERAL  
ADMINISTRATION OF ASCORBIC ACID

By BERNARD S. GOULD AND HARRY SHWACHMAN

WITH THE TECHNICAL ASSISTANCE OF JAMES F. LENNEY

(From the Department of Biology and Biological Engineering, Massachusetts  
Institute of Technology, Cambridge)

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In previous investigations (1, 2) the relationship between "alkaline" phosphatase<sup>1</sup> activity and ascorbic acid intake in experimental scurvy has been studied. It has been shown (1) that the serum phosphatase is of osteoblastic origin in the guinea pig and that in scurvy in which there is impaired osteoblastic activity the serum alkaline phosphatase level falls markedly. Upon the administration of ascorbic acid (0.25 to 12.0 mg.) there was shown to be a recovery of osteoblastic activity and a concomitant rise in the serum phosphatase level (2).

The present investigation has been undertaken to determine whether the histological changes involved in the scorbutic process can be equated to the enzymic change and in this manner serve as a quantitative bioassay method for ascorbic acid or any other antiscorbutic substance. Such a method would obviate the use of the non-specific growth methods or the use of the usual histological methods which are specific but require much time, complicated histological manipulation, and highly skilled interpretation. The new method would combine the simplicity of the growth method with the specificity of the histologic tooth structure method of assay.

On the basis of the present investigation a new method for the bioassay of ascorbic acid is suggested.

## Methods

*Animals*—Guinea pigs 6 to 7 weeks of age and weighing from 250 to 275 gm. (except for special experiments separately described) were separated into groups of five and housed in clean wire cages. The animals were fed *ad libitum* a scorbutogenic diet consisting of equal parts of sklm milk

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts Institute of Technology.

<sup>1</sup> Phosphatase, as used throughout the present communication, refers to the enzyme with an optimum activity at pH 8.6 to 9.0.

(heated at 100° for 4 to 5 hours), rolled oats, and bran. The diet was supplemented by 1 ml. of cod liver oil every 4 to 5 days. Fresh water was available to the animals at all times. Measured amounts of crystalline *l*-ascorbic acid, dissolved in water immediately before use, were administered by mouth when desired by means of accurately calibrated syringes. The scorbutogenic diet was adequate for normal growth when supplemented with sufficient amounts of ascorbic acid; without the supplement the animals invariably showed evidence of scurvy in 18 to 25 days. Larger animals required a longer time to develop scurvy.

*Estimation of Serum "Alkaline" Phosphatase. Blood Sampling*—The animals were bled directly from the heart under very light ether anesthesia. 0.5 to 1.0 ml. of blood is withdrawn into a small tube, allowed to clot, and the serum centrifuged off. Small samples can be used, since only 0.05 ml. of serum is needed for the phosphatase estimation. Light ether anesthesia and repeated small bleedings at short intervals were found to have no effect on the phosphatase level.

*Phosphatase Estimation*—In the present investigation, which has been adapted to the use of very small amounts of serum, a slight modification of the method suggested by Shinowara, Jones, and Reinhart (3) has been employed. The serum phosphatase may also be determined by the method of Bodansky (4), in which inorganic phosphate is estimated by the method of Fiske and Subbarow (5), but larger amounts of serum (0.5 ml.) are required and from 2.0 to 3.0 ml. of blood should be drawn.

0.05 ml. of the serum is diluted to 0.50 ml. with distilled water and two samples of 0.02 ml. each are withdrawn and introduced into Kahn serological tubes (12 × 75 mm.). The tubes are placed in a water bath, carefully adjusted to maintain the temperature at 37°, and allowed to remain for a few minutes. To one (the control) is added 0.4 cc. of distilled water and to the other 0.4 cc. of substrate made up as follows: 1.06 gm. of sodium diethyl barbiturate (veronal), Merck, 1.25 gm. of sodium  $\beta$ -glycerophosphate (Eastman Kodak), and 0.5084 gm. of  $MgCl_2$  in 250 ml. of distilled water. After the addition of serum the digest is at pH 8.6. After incubation for 1 hour, 0.4 ml. of 10 per cent trichloroacetic acid is added to each tube and the tubes centrifuged at 2000 R.P.M. for 10 to 15 minutes. The water-clear supernatant fluid is decanted into another tube and is ready for the analysis of inorganic phosphate, which is carried out as described (3). The difference between the inorganic phosphate levels in the presence and absence of sodium  $\beta$ -glycerophosphate is a measure of the phosphatase activity, which is expressed in units each of which is equal to 1 mg. of phosphorus (as phosphate) liberated by 100 ml. of serum in 1 hour at 37°, under the conditions of the experiment. It is desirable to check the substrate periodically for free inorganic phosphate and it is essential that

new calibration curves be made for each set of new reagents (to be published). A blank estimation should be run on the reagents without added inorganic phosphate. By this method repeated estimations indicated that an accuracy of  $\pm 5$  to 10 per cent was attainable.

#### EXPERIMENTAL

It has been found previously (2) that doses of ascorbic acid in quantities from 1.0 to 12.0 mg. per day appeared to be adequate to maintain the serum phosphatase levels (preventive method) and that ascorbic acid (0.25 to 12.0 mg.) administered to scorbutic animals with lowered phosphatase levels (curative method) resulted in prompt increases in serum phosphatase. What is more, there appeared to be no correlation indicated in those experiments between these doses and the response, the lower dosage (0.25 mg. per day) showing practically the same increases in magnitude and nature as the highest dose (12.0 mg. per day). Since lower doses than 0.25 mg. per day were not studied, the following experiments were undertaken.

*Preventive Method*—A series of experiments was carried out to determine whether a critical level of ascorbic acid is required to prevent a decline in serum phosphatase. For this purpose the animals were divided into several groups of eight animals each. All were fasted for 2 days and then put on the scorbutogenic diet. The diet of each group was then supplemented with either 0.00, 0.1, 0.15, 0.2, 0.225, 0.25, 0.3, 0.4 mg. of ascorbic acid per day (fed by mouth). At intervals the serum phosphatase of each group was determined. From typical results of several such experiments Figs. 1 to 4 have been constructed and indicate quite clearly that in doses of 0.225 mg. per day or greater the serum phosphatase is maintained at its normal level; below this dosage there is a sharp fall in the phosphatase level (Table I). There appears to be no direct relationship between the dosage under 0.225 mg. per day and the phosphatase level, which indicates that there is a critical intake required, amounts smaller than this having but slight effect on the phosphatase level.

The results of several experiments indicate that different groups of animals behave differently on the scorbutogenic diet. The rate of decline of the serum phosphatase differs, some groups attaining the low level rapidly (15 days), others slowly (25 to 30 days). The initial values for the phosphatase level before the animals are put on the diet vary considerably as well.

*Curative Method*—Several groups of five or ten animals each were put on the scorbutogenic diet for a period of from 15 to 25 days, depending upon the period necessary to bring the serum phosphatase down to a level of about 4 to 5 units. Lower levels were found not to be as desirable, since further drops in the enzyme level could not be studied accurately. When



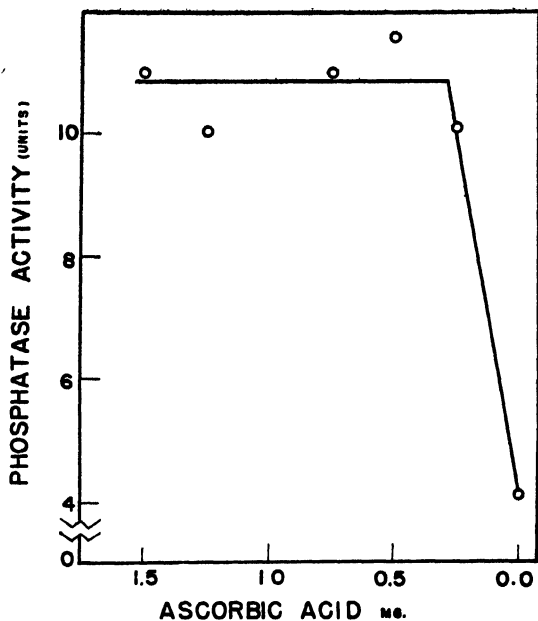


FIG. 1. The serum alkaline phosphatase of 250 gm guinea pigs fed a scorbutogenic diet supplemented with varying doses of ascorbic acid for 30 days.

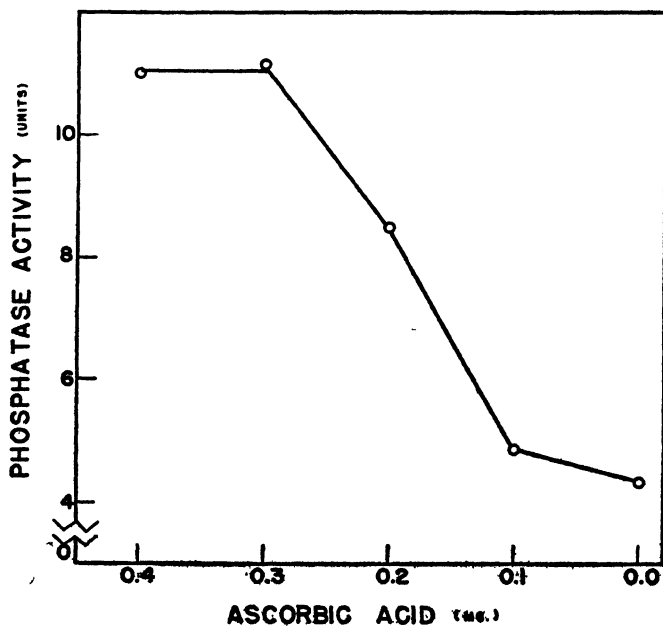


FIG. 2. The serum alkaline phosphatase of 250 gm. guinea pigs fed a scorbutogenic diet supplemented with varying amounts of ascorbic acid for 15 days.

the serum phosphatase had fallen to this range, varying doses of ascorbic acid (0.00, 0.10, 0.15, 0.175, 0.20, 0.225, 0.25, 0.275, 0.3, 0.35, 0.4, 0.5 mg. per day) were administered by mouth. After various intervals of from 3 to 15 days the serum phosphatase was estimated. From some typical data (Tables II and III) it is apparent that 0.225 mg. per day is a critical dose for a 5 day response and 0.2 mg. per day for a 10 day response. Doses

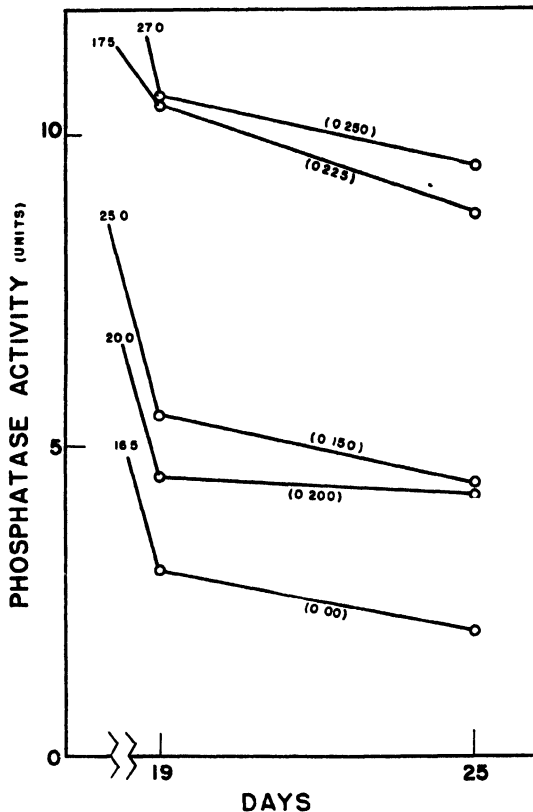


FIG. 3. The serum alkaline phosphatase of a group of 250 gm. guinea pigs fed a scorbutogenic diet supplemented with from 0 to 0.250 mg. of ascorbic acid (figures in parentheses). The initial phosphatase level is also indicated on each curve.

below 0.2 mg. were not very effective. Doses of 0.3 to 0.4 mg. per day result in responses in 3 days. If the curative period is carried to 15 days with doses of 0.2 and 0.225 mg., the phosphatase level begins to fall again, indicating that the range is critical, and as the animal becomes more scorbutic the dose cannot maintain the increase apparent at first (Table III). The critical level is taken as that dose at which most of the animals

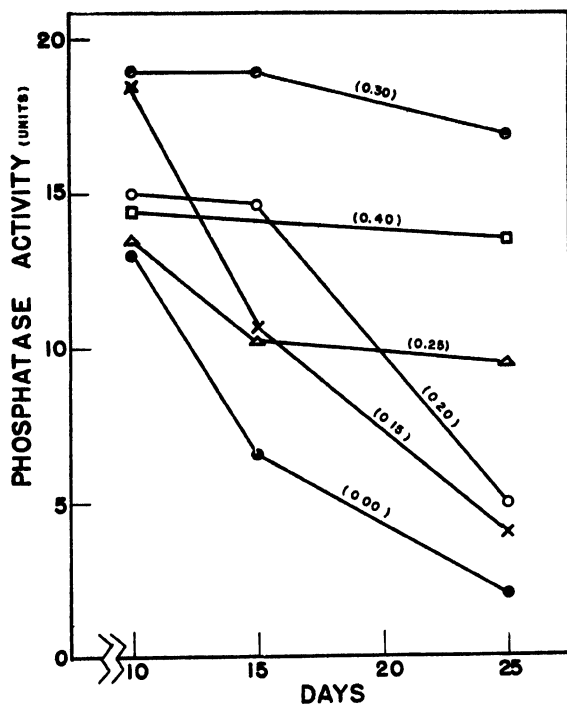


FIG. 4. The serum alkaline phosphatase of a group of 175 to 200 gm. guinea pigs fed a scorbutogenic diet supplemented with varying doses of ascorbic acid (figures in parentheses). The initial values varied from 16 to 35 units

TABLE I

*Typical Example of Effect on Serum Phosphatase Activity of Feeding Scorbutogenic Diet Supplemented by Relatively Small Amounts of Ascorbic Acid to Guinea Pigs*  
The results are expressed in phosphatase units.

Animal No.	Days on diet			
	0		19	
	0.20 mg. daily		0.225 mg. daily	
1	16.6	6.6	17.4	8.9
2	27.0	4.4	14.4	14.0
3	18.8	4.3	15.6	8.7
4	14.6	4.6	15.0	8.3
5	24.2	3.7	23.4	10.6
Average	20.2	4.7	17.2	10.1

TABLE II

*Influence of Varying Doses of Ascorbic Acid Fed Daily for 5 Days on Serum Phosphatase of "Scorbutic" Guinea Pigs*

The results are expressed in phosphatase units.

Dosage per day	0 time	5 days	Dosage per day	0 time	5 days	Dosage per day	0 time	5 days
mg			mg.			mg.		
0.15	5.9	5.3	0.225	3.1	8.4	0.25	3.7	8.7
	5.2	3.1		3.4	11.3		2.7	5.8
	7.9	4.8		3.8	4.2		4.5	11.4
0.175	3.2	2.9		4.6	9.7		4.2	6.2
	3.2	2.8		4.1	4.4		5.5	9.9
	4.4	4.9		5.2	12.2	0.3	4.3	12.3
0.2	4.1	6.3		4.3	4.1		5.0	9.6
	7.7	4.8		4.3	8.3		1.9	15.8
	3.3	2.0		2.4	4.1	0.4	4.7	15.8
	6.5	6.2		1.1	10.2		2.0	10.7
	3.9	5.2		3.1	5.3		1.7	6.7
	8.6	4.9		3.5	6.5		5.4	13.1
	5.6	6.2		2.0	7.4		1.0	9.5
	5.1	2.9		1.7	6.8		4.1	14.1
	5.4	5.9		4.7	7.7		4.5	16.1
				4.1	14.1			
				1.9	6.7			
				2.7	7.3			

TABLE III

*Influence of Small Doses of Ascorbic Acid on Serum Phosphatase Activity of Scorbutic Guinea Pigs*

The results are expressed in phosphatase units.

Dosage per day	Curative period				
	0 day	3 days	5 days	10 days	15 days
mg					
0.20	4.1	5.9	6.3	8.2	3.9
	7.7	5.3	4.8	11.0	6.4
	3.3	3.4	2.0	7.3	5.7
	5.3		4.9	11.8	
	5.1		2.9	7.8	
0.225	5.5	5.8	6.4	9.7	5.5
	5.4	7.1	10.6	12.2	5.5
	5.3	5.9	8.5	10.4	4.3
	5.7		9.9	13.3	

of a group show a sharp increase in phosphatase (Table IV), while animals in the group getting a lower dose show a drop in serum phosphatase. With

doses of less than 0.225 mg. per day all, or practically all, would show definite decreases or no change in enzyme level. Occasionally one animal of the group receiving the subminimal dose will show an increase; this is neglected. For any particular stock of animals the "minimal dose" must be determined since it is not unlikely that there would be slight variation from stock to stock. The 5 day period was found to be the most convenient, since animal variability played a less significant rôle than when the shorter periods were used.

Histological examination<sup>2</sup> of sections of the tibiae of animals on such experiments confirmed the observation that 0.2 to 0.225 mg. per day is a critical level since no significant healing could be detected in animals fed smaller doses, while after 5 days on 0.225 mg. daily the first definite effects became apparent. As would be expected, there were definite signs of

TABLE IV

*Typical Assay of Preparation of Unknown Potency*

Groups I to IV received doses which appeared to contain, on the basis of chemical titration, 0.2, 0.225, 0.25, and 0.275 mg. of ascorbic acid, respectively. The bioassay indicates that Group III received the critical dose of 0.225 mg. daily

The results are expressed in phosphatase units

Animal No	Group I		Group II		Group III		Group IV	
	0 time	5 days	0 time	5 days	0 time	5 days	0 time	5 days
1	3.1	1.6	2.3	0.3	2.5	6.1	1.5	8.2
2	4.6	0.8	1.6	2.8	4.0	8.8	1.8	8.4
3	5.4	4.2	3.8	2.3	3.4	6.6	3.6	10.1
4	0.8	Died	4.7	4.7	2.7	3.1	4.2	6.9
					Critical dose			

scurvy accompanied by slight healing and a concomitant rise in the phosphatase level. It would appear therefore that the change in phosphatase is one of the first stages in the correction of the pathological condition.

## DISCUSSION

*Comparison of Preventive and Curative Methods*—To adapt the changes in serum phosphatase that accompany scurvy or that result from the treatment of scurvy to a method for the bioassay of ascorbic acid, it was felt that the curative method offered greater possibilities than the preventive method. The curative method requires but 5 days, once a supply of "scorbutic" (low serum phosphatase) animals is available, while the preventive method involves a testing period of 18 to 24 days. Not only

<sup>2</sup> Kindly carried out by Dr. Sidney Farber of the Children's Hospital and Harvard Medical School, to whom we express our thanks.

does this mean that, with a supply of scorbutic animals on hand, the curative test is less time-consuming but that the chances of losing animals during the test period by inanition and intercurrent infection are negligible. This was found to be of considerable concern with the preventive method, since frequently a large percentage of a group was lost during the test period. For the curative method animals can be selected so that they have lowered phosphatase levels but are not in such poor condition that they cannot survive another 5 days. Doubtful animals should not be used. This assures better statistical data on the completion of an assay.

Frequently it is difficult to adapt guinea pigs to the powdered scorbutogenic diet and for several days they refrain from eating. During this period there is a sharp drop in serum phosphatase even if ascorbic acid is administered (2). This influences the preventive assay carried out on such animals. In the curative method animals are used which have already become adapted to the powder diet and therefore this variable is eliminated.

There is considerable variation among the animals with respect to the initial serum phosphatase level which varies from about 12 to 35 units (Figs. 3 and 4). There is also considerable variability in the rate of decline of the serum phosphatase level, in normal animals due to age and in animals on the scorbutogenic diet to the drop due to incipient scurvy superimposed on the normal decline due to aging. Some groups of animals on the scorbutogenic diet attain a level of 3 to 5 units in 15 to 18 days, while other groups do not reach this level until after 25 days. Variations such as these make it practically impossible to use the preventive method effectively.

*Factors Which Might Influence Curative Method. Fasting*—It was found in a previous study (2) that animals frequently refused to eat the powdered diet for several days and in spite of the administration of ascorbic acid showed sharp decreases in serum phosphatase. Usually, as has been pointed out, by the time the animals are ready for the assay they have learned to eat the diet. On the other hand, it is well known that the intake of the scorbutic animal is much smaller than that of the normal animal and this varies from animal to animal. It has been suggested by Bodansky and Jaffe (6) that fasting lowers the serum phosphatase of dogs; therefore an experiment was undertaken to determine whether fasting would influence the phosphatase response of scorbutic animals to ascorbic acid. Food was eliminated but water was available to the animals at all times. No differences in phosphatase response could be determined between normally fed and fasted animals. It appears therefore that the refusal of some animals to eat during the curative period would not influence the results of the bioassay.

*Cod Liver Oil Feeding*—In view of the observations (unpublished results) that the feeding of fat or cod liver oil to rats results in markedly elevated serum phosphatase levels and the withdrawal of fat leads to lowered levels (7), it was desirable to determine whether the withdrawal of cod liver oil from the preparative diet as well as from the diet during the curative period, or its excessive administration during these periods, would influence the serum phosphatase response to ascorbic acid. The results of such experiments indicated that there is no appreciable effect on the phosphatase response, although many of the animals suffered acute intestinal distress and died when excessive fat or cod liver oil was administered. On the other hand, the withdrawal of cod liver oil, which is a more probable occurrence than excessive fat feeding during the assay period, resulted in no difficulties which would influence the assay.

Since rickets is one of the few nutritional disturbances which are known to affect the serum phosphatase and since rickets is accompanied by an increase in the serum phosphatase level (8), an experiment was carried out to determine whether there were any abnormalities in phosphatase level in guinea pigs fed a rachitogenic diet (U.S.P. XI, Diet 2) during the preparative and curative periods of the assay. No complications were apparent.

*Age of Animals*—Experiments were carried out to determine the influence of the age of the test animal on the results of the assay, since it seemed likely that young animals might be prepared for assay in a shorter time. For most of the experiments 6 to 7 week-old animals weighing 250 to 275 gm. were used. In the following experiment animals of 4 to 5 weeks weighing 175 to 200 gm. were put on the same regimen as the larger animals and another group which had been reared on the scorbutogenic diet supplemented with ascorbic acid and had attained a weight of 400 to 450 gm. was also studied. This procedure was necessary for the larger animals, since normally reared large animals invariably refuse the powder diet. It was found that the smaller animals were not as desirable for assay purposes because of the variability of the initial phosphatase level, and the irregularity of the decline in phosphatase level accompanied by severe manifestations of scurvy, resulting in high mortality among the test animals. On the other hand, no difference in critical requirements was observed. The older animals (400 to 450 gm.) were found to require a very long preparatory period but eventually the phosphatase level fell to the desired point and the response was practically identical with that for the 6 to 7 week-old animals.

*Reuse of Animals*—In several instances animals which had been used for one assay were put back on the scorbutogenic diet for a period of 5 to 7 days and then used again for assay purposes. In this short preparatory period the phosphatase value usually fell to the desired level and the

animals served perfectly well for a second assay. On repeated assay with the same animals it was found that there was a slightly smaller requirement, 0.2 mg. instead of 0.225 mg. in a few instances.<sup>a</sup> It is suggested that animals not be used for *several* assays unless controls with pure ascorbic acid are run simultaneously.

*Specificity of Assay Method*—From previous work it was indicated that no scorbutic animal failed to show an increase in serum phosphatase after ascorbic acid therapy. Other conditions (8) are known to affect the serum phosphatase level and it was desirable to determine whether the administration of ascorbic acid would produce an increase in serum phosphatase in conditions other than scurvy. Since guinea pig material of this sort is limited, a large group of children suffering from various disorders at the Children's and Infants' Hospital, Boston, was studied. Cases of nutritional disturbance, especially, were selected. Serum phosphatase was determined before and after ascorbic acid therapy. In no condition other than scurvy was there an increase in serum phosphatase due to ascorbic acid treatment. It would appear that there probably are no common pathological conditions that would seriously influence the interpretation of the assay by the curative method, especially if adequate numbers of animals are used.

#### *Proposed Method*

A group of twelve to sixteen animals (6 to 7 weeks old, weighing 250 to 275 gm. at the start of the experiment) which have been on the scorbutogenic diet for a period of 18 to 25 days is selected and the serum phosphatase estimated and separately recorded for each. If the phosphatase levels are in the range of 3 to 5 units (animals with lower values may be used), the animals are divided into three or four groups of four animals each. (A greater number or larger groups may be used if desired. Groups of three to five animals have been found adequate.)

*Sample*—A chemical estimation of the probable ascorbic acid and dehydroascorbic acid content of the unknown is carried out by 2,6-dichlorophenol indophenol titration (9). Each animal of the respective groups is fed daily a quantity of the sample equivalent to either 0.2, 0.225, 0.25 mg. (and 0.275 mg. if a fourth group is used), as determined by the titration. On the 5th day after the first dose the animals are bled and the serum phosphatase determined.

*Interpretation*—Any groups in which 50 per cent or more of the animals shows a decrease in serum phosphatase and in which the other animals of the group show no significant increase have been receiving less than 0.225 mg. of ascorbic acid daily. If most of the animals show an increase and some show no extensive change, the group has been receiving the



critical dose of 0.225 mg. If all show marked increases, the critical level may have been exceeded. If only one animal shows an increase while all the others show a decrease or are unchanged it should be neglected. Increases of about 10 per cent should also be disregarded in view of the probable error of the estimation. A typical assay of a preparation which has antiscorbutic activity is shown in Table IV. An assay of an inactive preparation is shown in Table VI.

*Notes*—For the qualitative determination of antiscorbutic properties a quantity of sample containing 10 to 20 times the minimal dose of 0.225 mg. is administered daily to a group of three to four "scorbutic" animals and after 5 days the serum phosphatase is determined. There should be no increases in the phosphatase level if the sample is inactive.

A control assay with pure ascorbic acid should be carried out for any new stocks of animals. For best results, controls, three to four animals on doses of 0.2 and 0.225 mg. per day, should be included in each assay or each group of assays run simultaneously.

#### *Assay of Some Compounds for Antiscorbutic Potency*

To test the validity and specificity of the method for the assay of antiscorbutic substances a number of substances of known purity, known, and, in some cases, questionable potency were assayed by the newly devised curative method of assay. All figures are based on the results obtained usually with groups of five animals for each dosage tested. In cases of questionable substances larger groups of animals were used. At the same time that these assays were being carried out, pure *l*-ascorbic acid was repeatedly assayed.

*Dehydroascorbic Acid*—The antiscorbutic potency of dehydroascorbic acid has been the subject of considerable controversy. While it has been variously reported as having "practically the full potency" of the parent substance (10-12) few actual data are presented. King (personal communication) states that dehydroascorbic acid has been found to have "80 to 90 per cent and certainly not less than 75 per cent" of the activity of *l*-ascorbic acid. On the other hand, Roe and Barnum (13) found it to be only one-quarter as active. Borsook *et al.* (12) explain this discrepancy on the basis of the methods used by Roe and Barnum. In the present assay, dehydroascorbic acid was freshly prepared by iodine oxidation and administered daily immediately after preparation. Doses varying from one-half to twice the theoretical critical dose of *l*-ascorbic acid were given and it was found (Table V) that dehydroascorbic acid has 80 per cent of the activity of the pure vitamin. This is, therefore, in agreement with the assay as carried out by other methods.

*d-Glucoascorbic Acid*—This analogue of ascorbic acid was obtained from

the Eastman Kodak Company. Previously it has been reported as having no antiscorbutic activity, even in 40 times the minimal dose required of *l*-ascorbic acid (4). An assay was carried out with it in doses up to 40 times the critical level for *l*-ascorbic acid and it was found inactive (Table VI).

*d*-Isoascorbic Acid—This was a sample obtained from the Eastman Kodak Company. It has been reported as having one-twentieth the activity of *l*-ascorbic acid (14, 15). It was assayed by the new curative

TABLE V

*Summary of Assays of Various Substances and Comparison with Reported Antiscorbutic Potency*

Substance	Antiscorbutic potency, per cent of <i>l</i> -ascorbic acid	
	Reported	Found
<i>l</i> -Ascorbic acid	Full potency	Full potency
Dehydro- <i>l</i> -ascorbic acid	25 to about 100% (10-13)	80%
<i>d</i> -Isoascorbic acid	5% (14, 15)	5-7%
<i>d</i> -Glucoascorbic acid*	None (16)	None
2-Ketogulonic acid†	Questionable (17, 18)	"
Iron ascorbate	" potency (20)	Equivalent to ascorbic acid in complex, ca. 80%

\* Given in 40 times the critical dose for *l*-ascorbic acid.

† Given in 30 times the critical dose for *l*-ascorbic acid.

TABLE VI

*Effect on Serum Phosphatase of Feeding d-Glucoascorbic Acid,\* Having No Antiscorbutic Activity, to Scorbutic Guinea Pigs*

The results are expressed in phosphatase units.

Animal No	Curative period		
	0 day	5 days	10 days
1	3.8	1.9	1.6
2	6.6	4.5	2.3
3	5.5	6.3	1.6

\* A dose equal to 40 times the critical level of *l*-ascorbic acid.

method and found to have between one-fifteenth and one-twentieth the activity of *l*-ascorbic acid.

*2*-Ketogulonic Acid—This compound, which is an intermediate in the technical synthesis of *l*-ascorbic acid and is the stable reductant of 2,3-diketogulonic acid which results from the opening of the lactone ring of ascorbic acid, has been reported by Ball (17) as questionably antiscorbutic and has no ability to regenerate ascorbic acid; it has also been

reported by Lorenzini and Corbellini (18) as inactive. A sample of 2-ketogulonic acid was obtained (through the courtesy of Professor R. C. Hockett, Massachusetts Institute of Technology, to whom we express our thanks) and the amount of ascorbic acid present in the sample was determined by 2,6-dichlorophenol indophenol titration. There was such a small trace that up to 30 times the critical dose (6.75 mg.) could be given without administration of significant amounts of *l*-ascorbic acid. It was found on assay that even in this large dosage it has no apparent antiscorbatic activity. The presence of 2,3-diketogulonic acid in the preparations of dehydroascorbic acid of Roe and Barnum (13) probably explains the low potency reported by them for dehydroascorbic acid.

*Complex of Iron and Ascorbic Acid*—This compound known as "ferro-ascorbate" has been described (19) and has been reported as antiscorbatic (20). A sample was freshly synthesized (21) (with Stewart Roe) and carefully purified by repeated selective extraction so that it contained no free ascorbic acid. The black powder, which gave intensely blue solutions, was studied chemically (to be reported) and found to contain 18.5 per cent Fe. Solutions of the complex were freshly prepared immediately before administration and assayed according to the new method. It was found that the complex is antiscorbatic and that its activity is comparable to the amount of ascorbic acid in the complex.

#### *Effectiveness of Various Methods of Administration of Ascorbic Acid*

The assay method has been applied to a study of the relative effectiveness of oral and parenteral administration of ascorbic acid. The inactivation of ascorbic acid by intestinal bacteria *in vitro* is well known but few data are available on the loss of ascorbic acid when given by mouth. Hou (22) has reported that ascorbic acid given by mouth is one-half as active as when given subcutaneously.

In the following experiment four series of animals were studied. One series received varying doses by mouth as in the usual assay procedure, a second was injected subcutaneously, a third intraperitoneally, and a fourth intracardially. The results of the experiment suggest that there is an apparent loss of approximately 27 per cent when the vitamin is given by mouth, since the critical level of ascorbic acid required to maintain the phosphatase level when the vitamin is injected subcutaneously or intraperitoneally is only 0.165 mg. per day. The results of repeated assays on the animals given intracardial injections were extremely irregular. Many animals showed the same responses as those receiving subcutaneous injections but many showed no increase. This may perhaps be explained by assuming that excretion of ascorbic acid by some of these animals receiving the vitamin directly into the blood may be much more rapid

than when it is given orally, subcutaneously, or intraperitoneally. However, there is no experimental basis for this assumption at present.

#### SUMMARY

A new bioassay for ascorbic acid is described, based upon the increase in serum "alkaline" phosphatase of scorbutic guinea pigs observed after a critical dose of ascorbic acid is administered.

The various factors which might influence the assay have been studied.

Substances of known and questionable potency as determined by other methods, such as dehydroascorbic acid, *d*-glucoascorbic acid, *d*-isoascorbic acid, 2-ketogulonic acid, and iron ascorbate, have been assayed.

A study has been made of the relative effectiveness of oral and parenteral administration of ascorbic acid which suggests a loss of 27 per cent when the vitamin is fed by mouth.

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# THE CHEMICAL DETERMINATION OF THIAMINE AND COCARBOXYLASE IN BIOLOGICAL MATERIAL\*

By BENJAMIN ALEXANDER

(From the Medical Service and Medical Research Laboratories, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston)

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The identification of substances in urine which interfere with the Prebluda-McCollum reaction for thiamine and the development of procedures for their elimination made available a simple colorimetric method for the chemical measurement of urinary thiamine (1). The present work describes the results obtained with this method when applied to other biological material: feces, liver, muscle, dehydrated tissues, and cereal.

Some of the methods already available for the estimation of thiamine and cocarboxylase in such biological materials are based upon biological reactions. Thus, the effect of thiamine and cocarboxylase on carbon dioxide production by *Ätiozymase* (2, 3) and the effect of diphosphothiamine on the oxidation of pyruvate by *Bacillus delbrückii* (4) have been used to measure the vitamin and coenzyme. The thiamine in feces (5) and tissues (5)<sup>1</sup> has been determined also by measuring its effect on the fermentation of certain strains of yeast; in this method tissue cocarboxylase was first converted into free thiamine by incubation with taka-diastrase. Other methods involve chemical reactions (6, 7) in which thiamine and cocarboxylase are oxidized to thiochrome and thiochrome diphosphate, the fluorescence of which is measured by a fluorometer. Free thiamine and its phosphorylated forms are differentiated by their different solubility in isobutyl alcohol.

The difficulties of, and objections to, both the biological and thiochrome methods of thiamine measurement have been discussed in detail in an earlier communication (1). It should be noted that much of the confusion concerning the exact relation between thiamine and cocarboxylase in biological materials has arisen out of the use of biological methods of assay (8, 9).

More recently, thiamine has been measured by means of the Prebluda-McCollum reaction or other chemical reactions in which thiamine is coupled with diazotized organic compounds. This reaction has been applied to urine (1, 10), milk (11), sweat (12), and extracts of rice polishings,

\* This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

<sup>1</sup> Ferrebee, J. W., *et al.*, personal communication.

wheat germ, yeast, and liver (13). The complexities involved in extraction and elimination of interfering substances in the methods previously used have greatly limited their usefulness. Consequently, it was considered desirable to study the applicability to these materials of the simplified method already developed for measuring thiamine in urine (1).

### Method

*Thiamine and Cocarboxylase in Fresh Tissue*—Analyses were made on liver, kidney, and muscle of dogs, some of which received daily intramuscular injections of thiamine hydrochloride in amounts of 2.0 mg. per kilo for from 4 to 10 days before they were sacrificed. All determinations were on tissue biopsies or on material taken immediately after death from bleeding or air embolus.

For the measurement of free (non-phosphorylated) thiamine, an aliquot of tissue (5 to 20 gm.) was placed in approximately 150 cc. of boiling dilute hydrochloric acid and boiled for a few minutes in order to inactivate tissue phosphatases. The tissue was then minced and thoroughly ground in a mortar or dispersed in a Waring blender. The mixture was adjusted to a pH of approximately 2 with dilute hydrochloric acid and incubated overnight at 40° with 1.0 gm. of pepsin; longer incubations were found unnecessary. The mixture, which was almost completely liquefied by the pepsin, was analyzed by the procedure previously reported for urine (1), except that a single treatment of the material with Super Filtrol was always sufficient to insure complete adsorption of the thiamine.

"Cocarboxylase" determinations were made on an aliquot of the same tissue which was prepared in the usual way. The pH was adjusted to 4.5 to 5.0, and taka-diaxylase,<sup>2</sup> 0.1 gm. per gm. of fresh tissue, was added. The mixture was incubated overnight at 40°. In this way phosphorylated thiamine, which cannot be measured directly by adsorption and coupling was diazotized *p*-aminoacetophenone (10), is converted quantitatively into non-phosphorylated thiamine which can be measured together with the already existing free thiamine. The phosphorylated thiamine, which we term cocarboxylase (thiamine monophosphate has not been shown to exist in animal tissue), represents the difference between the values for the free and total thiamine thus obtained.

*Total Thiamine in Dehydrated Tissues<sup>3</sup> and Cereals*—Dried powdered

<sup>2</sup> Clausen and Brown (14) claim that taka-diaxylase may, under certain conditions, give incomplete splitting of cocarboxylase and that Polidase (Schwartz Laboratory, Inc., New York) is better. Under the conditions of our experiments, taka-diaxylase splits a pure solution of cocarboxylase completely and equals the splitting power of Polidase on cereals.

<sup>3</sup> We are indebted to Dr. C. A. Elvehjem who kindly furnished us with samples of dehydrated tissues.

tissue was suspended in dilute hydrochloric acid and finely dispersed in a Waring blender. Some determinations were made after the tissue had been subjected to digestion with pepsin. Incubation with taka-diestase was carried out in all analyses in the usual manner. Cereals were handled in the same way, except that the samples were cooked in dilute hydrochloric acid for 10 minutes before incubation with pepsin and taka-diestase. Longer heating was found unnecessary.

*Thiamine in Feces*—Human feces were collected in a jar containing 40 cc. of 10 per cent hydrochloric acid and 10 cc. of toluene. The total 24 hour stool was transferred to a mortar and was thoroughly dispersed in 300 to 800 cc. of water. The mixture was weighed and stirred, and an adequate aliquot (by weight) was taken for analysis. This was transferred quantitatively to a 250 cc. centrifuge flask, and sufficient water was added to make an approximate volume of 100 cc. The mixture was then centrifuged, and the supernatant was decanted into another 250 cc. centrifuge flask. The centrifugate was thoroughly washed twice with a total of 50 cc. of water, and the washings were combined with the original extract. The thiamine determinations were then made as previously described for urine (1).

### Results

*Critique of Method*—Preliminary experiments indicated that grinding tissues in a mortar or fine dispersion with a Waring blender resulted in maximal extraction of the thiamine. Grinding the tissue with sand gave no increase in thiamine values.

Duplicate analyses on aliquots of fresh and dehydrated tissues and on samples of feces and cereals showed good reproducibility (Table I).

Dehydrated tissue was used to see whether there was any relation between the amount of tissue analyzed and the apparent thiamine concentration or between the amount of known thiamine added and the total thiamine detected (Table I). The amount of thiamine obtained per gm. of tissue appeared independent of both the amount of tissue taken for analysis and the amount of known thiamine added to an aliquot. Furthermore, the density of color obtained by analysis of multiples of the same tissue sample, when plotted against the amount of tissue taken, could be exactly superimposed on a similar curve obtained with multiple volumes of a standard thiamine solution.

Pepsin has been used by other workers (7) to insure complete liberation of thiamine from tissue protein to which it might be bound. Experiments in which the thiamine measurements after pepsin digestion of the tissue were compared with analyses on aliquots of the same tissues without pepsin digestion throw doubt on the necessity for such treatment (Table II). Nevertheless, some of the following data for free thiamine have been



obtained with pepsin digestion of the tissue before analysis. Except where indicated, such treatment was omitted in analyses for total thiamine,

TABLE I  
*Duplicability of Thiamine Determinations*

Material	Weight	Thiamine chloride per gm	Deviation from mean	Material	Weight	Thiamine chloride per gm.	Deviation from mean
	gm.	γ	per cent		gm	γ	per cent
Fresh dog tissue				Cereal			
Liver	9.1	1.20	+4.7	Ralston	2.0	4.2	-12.5
	8.9	1.33	+7.1		3.0	4.4	-7.5
	10.8	1.16	-7.9		4.4	5.0	+5.0
	8.1	1.35	-7.1		4.4	5.1	+7.5
Muscle	11.3	0.62	-1.6		4.4	5.0	+5.0
	11.3	0.64	+1.6		4.4	5.1	+7.5
Liver*	4.6	3.04	+1.3		4.4	4.9	+2.5
	4.6	2.86	-5.3		4.1	4.4	-7.5
	4.6	2.82	-6.0		5.0	4.9	+2.5
	4.6	3.07	+2.3		5.0	4.9	+2.5
	4.6	2.90	-3.3	Cerevim†	1.0	16.6	-9.8
"	9.6	2.50	+1.0		1.0	18.9	+2.7
	7.8	2.45	-1.0		1.0	19.1	+3.8
"	10.2	1.64	-0.6		1.0	19.1	+3.8
	11.8	1.66	+0.6	Human feces			
Muscle*	7.6	1.81	+5.8	Sample 1		1.86	+5.0
	6.0	1.61	-5.8			1.71	-5.0
Dehydrated tis- sue				" 2		1.06	-2.8
Pork loin	0.2	16.3	+5.4			1.12	+2.8
	0.5	15.0	-3.1	" 3		1.04	-2.8
	1.0	15.4	0			1.10	+2.8
	1.0	16.4	-6.1				
	2.0	15.6	+0.8				
	2.0	15.7	+1.6				
	2.0	14.4	-6.9				
	4.0	14.4	-6.9				
" ham	1.0	24.0	+2.5				
	2.0	22.8	-2.5				
Veal	2.0	1.07	-9.1				
	2.0	1.07	-9.1				
	4.0	1.55	+32.0				
	4.0	1.01	-13.1				

\* The animals received thiamine intramuscularly for several days before biopsy or death.

† Lederle Laboratories, Inc.

since incubation with taka-diastrase resulted in satisfactory liquefaction of the tissue, and preliminary pepsin digestion failed to increase the yield of thiamine.

The recovery of added thiamine<sup>4</sup> for kidney, liver, and dehydrated tissues was between 80 and 110 per cent (Table III). The somewhat lower yields in some analyses of muscle are difficult to explain in view of the excellent results of similar experiments on dehydrated muscle tissue;

TABLE II  
*Effect of Pepsin Digestion on Measurable Tissue Thiamine*

Tissue	Thiamine content	
	With pepsin	Without pepsin
	$\gamma$ per gm.	$\gamma$ per gm.
Dog liver*.	1.16	1.20
" " †.	1.35	1.33
" muscle*.	0±	0.13
" " †.	0.89	0.70
" " †.	0±	0.04
Pork, dehydrated*	22.8	24.0
Veal, " *	1.56	1.07
	1.01	1.07

\* Analysis for total thiamine.

† Analysis for free thiamine.

TABLE III  
*Recovery of Added Thiamine*

	Material	No. of determinations	Amount of material	Recovery of added thiamine (26 $\gamma$ ); No of determinations			
				90-110 per cent	80-90 per cent	70-80 per cent	60-70 per cent
			gm				
Fresh tissues	Dog liver	25	3.3-46.0	14	11	0	0
	" kidney	13	2.0- 7.8	9	4	0	0
	" muscle	19	5.0-24.0	4	7	6	2
Dehydrated tissues	Pork loin	7	0.2- 2.0	7	0	0	0
	" ham	2	1.0- 2.0	2	0	0	0
	Veal	3	2.0- 4.0	3	0	0	0
	Beef	1	5.0	1	0	0	0
Cereals	Ralston	8	2.0- 5.0	7	1	0	0
	Cerevim*	4	1.0	3	1	0	0
Feces		23	1.0-15.0†	5	13	4	1

\* Lederle Laboratories, Inc.

† Feces of two patients who received thiamine orally.

possibly the removal of fat in the process of dehydration of these tissues may account for the better yields. The slightly lower recoveries on some

<sup>4</sup> Thiamine hydrochloride was added immediately after the tissues had been ground and dispersed.

specimens of feces may also be due in part to their relatively high fat content. The recovery of thiamine added to fresh tissue, cereals, and

TABLE IV  
*Tissue Thiamine and Cocarboxylase in Dogs*

Tissues	Free thiamine $\gamma$ per gm	Cocarboxylase $\gamma$ per gm.	Total thiamine $\gamma$ per gm.
Liver.. . . .			0.60
" . . . . .			0.74
" . . . . .			0.51
" .. . . .			0.39
" . . . . .			1.26 (0.70)
Muscle ... . .			0.35
" . . . . .			0.62
" . . . . .	0.07	0.49	0.56 (0.51)
Kidney... . .	0.21	1.58	1.79

Thiamine administered parenterally daily for several days before analyses			
Liver.. . . .			1.80
" . . . . .			2.13
" .. . . .			2.69
" . . . . .	0.55	3.29	3.84
" . . . . .	0.13	2.47	2.60
" .. . . .	0.27	1.44	1.71
" . . . . .			2.70
" . . . . .	0±	1.52	1.52
" .. . . .	0.11	1.97	2.08
" . . . . .	0.21	1.80	2.01
" . . . . .	0.16 (0.20)	1.48 (2.00)	1.64 (2.25)
Muscle . . . . .			1.50
" . . . . .	0.28	1.45	1.73
" . . . . .	0.07	2.13	2.20
" . . . . .			1.70
" . . . . .	0±	2.56	2.56
" . . . . .	0.28	2.74	3.02
" . . . . .	0.10 (0.15)	1.60 (2.10)	1.70 (2.06)
Kidney . . . . .	0.50	2.87	3.37
" . . . . .	0.79	1.71	2.50
" . . . . .			2.35
" . . . . .	0±	2.24	2.24
" . . . . .	0.14	1.96	2.10
" . . . . .	0.52	1.41	1.93
" . . . . .	0.35 (0.38)	1.87 (2.01)	2.22 (2.39)

The figures in parentheses represent the averages.

feces appeared to have no relationship to the amount of material taken for analysis.

*Thiamine and Cocarboxylase Content of Tissue*—The total thiamine content of dog kidney, liver, and muscle (Table IV) averaged respectively 1.79, 0.70, and 0.51  $\gamma$  per gm. of fresh tissue of animals which had not received supplementary amounts of the vitamin. This is in agreement with the observations of others. The values were much higher in animals given daily intramuscular injections of 2.0 mg. of thiamine per kilo for several days before analysis. The muscle and liver values were about 3 to 4 times greater than those of untreated animals, and the values in all three tissues examined were about equal to one another.

The free, or non-phosphorylated, thiamine of fresh dog tissue represented 7 to 16 per cent of the total thiamine. The ratio of free to total thiamine was greatest in the kidney.

The analyses on dehydrated tissues (Table I) show an extremely high thiamine content for pork ham and a fairly high value for pork loin. The

TABLE V  
*Comparative Thiamine Analyses by Thiochrome and Colorimetric Methods*

Material	Thiamine content	
	Thiochrome method	Colorimetric method
	$\gamma$ per gm.	$\gamma$ per gm.
Pork ham, dehydrated . . . . .	25.6*	23.5
“ loin, “ . . . . .	17.3*	15.3
Ralston cereals . . . . .	5.8	4.8
Cerevim . . . . .	21.2	19.1
Standard thiamine solution. ....	20.0*	20.0

\* Analyses by Professor Elvehjem.

values for veal are much lower. Although these results are in agreement with those of other observers who find that pork loin and ham are excellent sources of thiamine, the actual values are approximately 10 per cent lower than those obtained on the same tissues by Elvehjem<sup>5</sup> and his group, who employ the thiochrome method (Table V).

*Thiamine Content of Feces*—Human feces were found to contain appreciable amounts of thiamine (Table VI). In one normal subject whose intake of thiamine was between 1.0 and 2.0 mg. per day, two 24 hour stools contained a total of 0.20 and 0.32 mg. of thiamine. The 24 hour stools of a patient deficient in the vitamin B group showed only 0.027 mg. of the vitamin. Following a 5.0 mg. dose given orally, the output of thiamine in the stool increased considerably. In two subjects who received 50 mg.

<sup>5</sup> We wish to express our gratitude to Professor Elvehjem for his cooperation in these comparative studies.

of thiamine parenterally daily the stools showed even larger amounts of thiamine.

*Thiamine Content of Cereals*—Total thiamine determinations on Ralston and Cerevim gave results slightly lower than the values claimed for these products by their manufacturers (Table V), who employed the thiochrome method. The discrepancies were of the same order of magnitude as those obtained on dehydrated tissues by the two methods.

TABLE VI  
*Thiamine in Human Feces*

Sample No.	Subject	Supplementary thiamine	Daily thiamine output	Diagnosis
		mg.	mg.	
1	H.	None	0.03	Vitamin B deficiency, carcinoma of bowel
2	"	5.0 mg. orally	0.26	Same
3	M.	None	0.32	Normal
		"	0.20	
		50 mg. parenterally	0.82	
4	L.	50 " "	0.42	"

#### DISCUSSION

The Prebluda-McCollum reaction, in which thiamine is coupled with *p*-aminoacetophenone, can be used for the determination of thiamine in various biological materials such as urine, feces, animal tissues, and cereals. Naturally occurring substances such as uric acid and ascorbic acid which interfere with this reaction can be readily eliminated (1), thus making possible a simple procedure whereby eight or more analyses may be carried out in 1 day. The duplicability of determinations by this method is within a deviation of  $\pm 8$  per cent from the mean, except where very small amounts, *i.e.* less than  $2 \gamma$  of thiamine, are to be measured; in such instances larger amounts of material for analysis may be taken. The analytical values for thiamine were independent of the amount of material analyzed within the limits described, and the recovery of added thiamine was in most instances between 80 and 110 per cent. For those materials (fresh muscle and feces) in which recovery of added thiamine may occasionally be lower, a correction factor may be applied for obtaining the true thiamine content.

The discrepancies of about 10 to 15 per cent between the analytical results obtained on the same material by this method and by the thiochrome method are difficult to explain. While there is a slight possibility that the differences are within the limit of error of the methods, the differences

are remarkably constant and in the same direction even for such diverse materials as dehydrated tissues and cereals. The results obtained on a standard thiamine solution by the two methods were identical. Since the results by this method on biological materials are lower than those obtained by the thiochrome method, the question arises whether non-thiamine fluorescent substances resulting from oxidation with ferricyanide are included in analyses by the thiochrome method. In this connection it should be pointed out that certain investigators (7) found that unless pepsin digestion was used before analysis by the thiochrome method, large blanks were obtained, presumably from the protein in the tissues.

Objection might be made that inadequate extraction of the thiamine from the material analyzed may account for our lower values. Opposed to this objection is the observation that grinding cereals to a very fine powder did not increase their thiamine values. Also, predigestion of the tissues with pepsin failed to give larger thiamine figures.

Since phosphorylated thiamine is not measured by the Prebluda-McCollum reaction as carried out in this procedure, the determination of both the free and phosphorylated vitamin may be accomplished by simultaneous analysis of an aliquot of the same material which has been subjected to enzyme hydrolysis. In the analysis for free thiamine, care must be exercised to destroy naturally occurring phosphatases which may, by splitting cocarboxylase before analysis, give values for free thiamine which are erroneously high. This precaution is particularly necessary when attempts are made to establish the true ratio between free and phosphorylated thiamine in biological materials.

The small ratio of free to total thiamine in animal tissues is in agreement with the findings of other observers (7) and lends support to the belief that the physiologically active form of the vitamin is its phosphorylated product. Some investigators (3), however, have reported a larger ratio in pigeon and rat muscle. This can be attributed to naturally occurring phosphatases in these tissues which might have split the cocarboxylase before analysis. Such splitting was easily demonstrable in a specimen of fresh dog muscle which, when minced and allowed to stand several hours at room temperature, showed all of its thiamine to be in the free form.

Other observers have shown that when thiamine is administered parenterally it is carried to the liver and other tissues, where it is rapidly converted into cocarboxylase. Although the data presented above in support of this finding are too meager to satisfy the criteria for statistical confirmation, our experiments suggest that not only are the thiamine values higher in the tissues of animals which receive supplementary thiamine, but also muscle, kidney, and liver in such animals are approximately equal in their thiamine content. Clearly, this relationship between the thiamine intake

and the tissue thiamine concentration might account for the wide variation in the analytical values reported for certain tissues by different observers under different conditions. An important corollary of this inference is that the calculated thiamine content of a diet, based upon reported food values, may be subject to enormous error.

The data also indicate the limited extent to which thiamine may be stored in the body. The daily administration of 2.0 mg. of thiamine per kilo to dogs for from 3 to 8 days before analysis failed to increase the tissue thiamine content to much more than 2  $\gamma$  per gm. It is clear that a very large part of the injected thiamine is either rapidly excreted or broken down in the body.

The presence of appreciable amounts of thiamine in feces indicates the need for taking into consideration losses via this route of excretion in balance studies of thiamine requirements. Although some fecal thiamine may arise from thiamine-synthesizing organisms in the gastrointestinal tract (15), most of it is probably orally ingested thiamine which has not been absorbed. Evidence in support of this is the observation that the oral administration of 5.0 mg. of thiamine will result in an increase in fecal thiamine excretion. A similar increase also follows administration of large doses of parenteral thiamine (50 mg. per day). This is probably a consequence of "flooding" of the body with the vitamin.

Thiamine assay by animal feeding may also be misleading, in view of the occurrence of unabsorbed thiamine in feces which may indicate incomplete "extraction," even in the gastrointestinal tract. Furthermore, increasing evidence of functional interrelationships between various members of the B complex and other vitamins makes it imperative that assay by feeding be simultaneously and adequately controlled by the administration of the same material, except for the substance under analysis.

#### SUMMARY

1. The Prebluda-McCollum reaction for thiamine has been applied to the determination of the thiamine content of animal tissue, grain cereals, and human feces.

2. By simultaneously measuring the free and total thiamine on aliquots of the same material, a value for the phosphorylated or cocarboxylase fraction may also be obtained.

3. Only very small amounts of free thiamine can be found in animal tissue and it represents but a small portion of the total thiamine.

4. The concentration of total thiamine and cocarboxylase in the tissues can be increased somewhat by the parenteral administration of this vitamin.

5. Significant amounts of thiamine are present in human feces and these amounts may be increased by the oral or parenteral administration of this vitamin.

6. The thiamine values obtained by this method are between 10 and 15 per cent lower than those obtained by the thiochrome method.

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# FORMATION OF A NICOTINAMIDE-LIKE SUBSTANCE FROM VARIOUS AMINO ACIDS AND RELATED COMPOUNDS

By MARIANNA R. BOVARNICK

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

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It was reported previously (1) that neutral solutions of asparagine and glutamic acid that have been heated for several days at 100° can replace nicotinamide as a growth factor for certain microorganisms. It was later reported that biological and chemical assays of the partially isolated active reaction product indicated that this substance probably is nicotinamide (2). In the hope of elucidating the mechanism of this unexpected reaction, further studies as to the most favorable conditions for carrying out the reaction and as to the ability of various other compounds to replace either of the reactants were undertaken and are presented in this paper.

## EXPERIMENTAL

*Assays for Nicotinamide Equivalent*—These assays were performed as previously described (1), by use of the growth and acid production of a strain of *Bacterium dysenteriae* Schmitz, No. 3822, on a synthetic medium containing asparagine, tryptophane, cystine, glucose, salts, and known amounts of nicotinamide as the standard. The measurement of both turbidity and pH allows the estimation of a wider range of concentrations than would the measurement of either alone, since the former is more sensitive to low concentrations and the latter to higher ones. The reproducibility of the results obtained with nicotinamide itself was not much greater than  $\pm 15$  per cent in separate experiments, and the accuracy of the assays may be even less when only slightly active reaction mixtures are tested, because some of the other substances present in the solutions tested affected the growth even in the presence of optimal amounts of nicotinamide.

The results of this method of assay are quantitatively significant only if nicotinamide, not nicotinic acid, is always formed in the reaction, since the response of these microorganisms to the acid is quite different from their response to the amide, as has been noted by other investigators (3). Under the conditions used, detectable growth occurs with the amide at a concentration of 0.002  $\gamma$  per ml. and increases slowly with increase in concentration to a maximum at about 0.02 to 0.03  $\gamma$  per ml. On the other hand, with the acid, growth is not detectable below a concentration of 0.015  $\gamma$  per ml. (7 times that required for the amide), then rises abruptly to a maximum at nearly the same concentration as with the amide, 0.02 to 0.03  $\gamma$  per ml.

In general, the concentration-growth curves of the solutions tested resemble more closely those for the amide. Therefore, the results have been expressed in terms of nicotinamide.

*Chemicals*—In most cases commercial compounds were used without further purification. Whenever possible, synthetic rather than natural amino acids were chosen. All the *dl* compounds mentioned in Tables II and III are synthetic; the optically active ones are obtained from natural sources. Asparagine was recrystallized as previously described (1). Glutamic acid was treated with charcoal and twice crystallized from water and alcohol.  $\alpha$ -Ketoglutaric acid was prepared as described by Neuberg and Ringer (4). The author is indebted to Dr. Marjorie Roloff of Columbia University for a generous sample of synthetic proline and to Dr. David Rittenberg of Columbia University for the oxalacetic acid and sodium diethyloxalacetate.

*Formation of Nicotinamide Equivalent*—Equimolar quantities of the two substances to be tested for their ability to form nicotinamide equivalent were weighed into  $18 \times 175$  mm. test-tubes calibrated at 25 ml., and were dissolved in water. The solution was neutralized with sodium hydroxide, with brom-thymol blue as an indicator, and made up to the desired volume. In some experiments given in Table III ammonia was used to neutralize the solution. Generally, the concentration of each reactant was 0.34 M and the total volume 5 ml. Except where otherwise noted, 0.1 ml. of 0.5 per cent  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.15 ml. of 0.5 per cent  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  were added for each 5 ml. of solution. In certain experiments designed to determine the effect of metal salts on the rate of the reaction between asparagine and glutamic acid, the salts added were varied as indicated in Table I. The tubes were fitted with finger condensers and placed in a constant level, boiling water bath for 48 hours. At the end of this period, the solutions were diluted to 25 ml. and autoclaved for 15 minutes. Suitable quantities were assayed for nicotinamide activity, at least two different concentrations that gave less than maximal growth being used when possible for each solution.

The results are expressed in terms of micromoles of nicotinamide produced per mole of amino acid. Although it has not yet been actually proved that nicotinamide is the active substance in all cases, this method of presenting the results has nevertheless been selected as the simplest means of comparing the amount of growth factor for *Bacterium dysenteriae* formed in the different mixtures.

The highest concentration of each amino acid tested in the final medium was 0.02 M. When tested at this concentration (0.02 M), formation of 8 micromoles of nicotinamide per mole of amino acid will permit full growth. Since asparagine or glutamic acid, after being heated alone for 48 hours,

may give growth equivalent to as much as 1.5 micromoles per mole of amino acid, formation of anything less than 2 micromoles per mole is probably insignificant. All reaction mixtures that gave little or no growth were tested also in the presence of added nicotinamide to make sure that inhibitory substances were not present. Whenever significant growth was noted in the absence of nicotinamide, the experiment was repeated with a different sample of the compound when available. In only one case was there any qualitative discrepancy; natural leucine appeared to yield nicotinamide on heating with asparagine, whereas the synthetic compound did not. This was probably due to the usual contamination of the natural compound with methionine, the most active of all the amino acids in this reaction. Quantitatively, the results were, however, less reproducible. The yield of nicotinamide from any given pair of reactants in duplicate experiments, performed at the same time and in the same water bath, showed a variation of about  $\pm 20$  per cent, but in the experiments performed on different days it sometimes varied from 2- to 3-fold. This variability is probably due to factors governing the reaction velocity, that are not yet understood.

### *Results*

The effect of the addition of certain salts on the velocity of the reaction between asparagine and glutamic acid is given in Table I. It can be seen that the quantity of nicotinamide formed in 48 hours is increased about 3-fold by the addition of iron and nearly 10-fold by manganese. The effect of copper is probably insignificant; magnesium, calcium, aluminum, chromium, cobalt, and nickel also had little effect. The increase in rate brought about by iron is believed to be significant, in spite of the fact that variations as great as this were obtained in experiments performed at different times, since in several different sets of experiments performed at the same time, the rate in each set was always greater in the presence of iron than in the absence of any metal and was always still greater in the presence of manganese. In subsequent experiments, both iron and manganese were added to the reaction mixtures, but it is probable that manganese alone is as effective as the combination.

Oxygen is another factor of importance in determining the rate of the reaction. This was noted especially in larger scale experiments run for much longer periods in order to obtain as high a yield of active substance as possible. With a volume of 1 liter, other conditions being the same as in the smaller scale experiments, heating for 14 days led to the formation of 360 micromoles of nicotinamide per mole of asparagine, whereas with aeration with oxygen for the first 4 days, then air, a 9 day reaction period gave yields of from 1200 to 2000 (average 1600) micromoles per mole. The necessity for oxygen might have been predicted from the fact that consid-

erable oxidation must occur in the formation of nicotinamide from asparagine or glutamic acid and neither compound would be expected to function as an oxidizing agent. This is also consistent with the effectiveness of iron as a catalyst, although it is less usual to find manganese a good catalyst for oxidations involving molecular oxygen.

Two types of compounds, the naturally occurring amino acids and certain 4- and 5-carbon dicarboxylic acids, were tested for their ability to replace either asparagine or glutamic acid in the production of a nicotinamide equivalent. The results obtained with the amino acids are given in Table II. All of these reaction mixtures were neutralized with sodium hydroxide. None of the amino acids listed forms a significant quantity of nicotinamide when heated with glutamic acid. This need not imply that asparagine is irreplaceable, since it may be recalled that isoasparagine, heated with glutamic acid, and glutamine alone were previously found to give some nicotinamide activity (1). These earlier results are not quantitatively

TABLE I

*Effect of Metal Salts on Rate of Formation of Nicotinamide from Asparagine and Glutamic Acid*

Salt added	Nicotinamide produced in 48 hrs.
	<i>micromoles per mole</i>
None . . . . .	29
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.015%	87
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.015%	260
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.015%	41

comparable with the others, as the experiments were performed under less favorable conditions.

In contrast to the limited number of closely related amino acids capable of replacing asparagine is the wide variety that can replace glutamic acid, although only methionine is as active in the reaction as glutamic acid itself. The compounds in Table II are listed in order of decreasing ability to form nicotinamide equivalent when heated with asparagine.

The results of the experiments with the dibasic acids are presented separately in Table III, since another variable was introduced in testing these compounds. In some cases, as indicated, the reaction mixtures were neutralized as usual with sodium hydroxide, in others with ammonia. In several instances the results varied with the base used for neutralization. The ammonium salts of fumaric, maleic, glutaric, and  $\alpha$ -ketoglutaric acids formed about twice as much nicotinamide as did the sodium salts when heated with asparagine. This difference is believed to be significant, since the same ratio was found when the two salts of glutaric and  $\alpha$ -ketoglutaric

acids were tested at the same time. The slight increase in the rate of the asparagine-glutamate reaction when ammonia is used for neutralization may be insignificant, since only one of the two experiments showed a yield larger than the highest found when sodium hydroxide was used.

TABLE II

*Formation of Nicotinamide Equivalent from Amino Acids Heated with Asparagine or Glutamic Acid*

Amino acid	Nicotinamide produced in 48 hrs				
	Alone	With glutamic acid	With asparagine		
			Average	No. of experiments	Range
	<i>micromoles per mole</i>	<i>micromoles per mole</i>	<i>micromoles per mole</i>		<i>micromoles per mole</i>
<i>l</i> -Asparagine	1.0*				
<i>l</i> -Glutamic acid . . . . .	1.0†		210	6	130 -270
<i>dl</i> -Methionine	1.2	1.8	250	3	150 -350
<i>dl</i> -Proline	<1	1.4	100	3	50 -140
<i>dl</i> -Citrulline	<1	<1	66	2	>10 - 66
<i>dl</i> -Ornithine hydrochloride	<1	2.4	52	2	48 - 55
<i>l</i> -Arginine hydrochloride	<1	1.0	13	2	12 - 14
<i>dl</i> -Phenylalanine	<1	1.2	13	2	10 - 17
<i>l</i> -Hydroxyproline	<1	1.2	10	2	8 - 11
<i>l</i> -Tyrosine	<1	1.4	10	3	8.8- 11
<i>dl</i> -Lysine hydrochloride		1.1	8	3	5.7- 12
<i>dl</i> -Serine	<1	1.6	8	3	7 - 10
<i>dl</i> -Threonine	<1	<1	5	3	3 - 10
<i>dl</i> -Alanine		<1	<1		
<i>dl</i> -Aspartic acid		1	<1		
<i>l</i> -Cysteine hydrochloride		<1	<1		
Glycine		1	<1		
<i>l</i> -Histidine hydrochloride		<1	<1		
<i>dl</i> -Isoleucine		<1	<1		
<i>dl</i> -Leucine		<1	1.1		
<i>dl</i> -Norleucine		<1	<1		
<i>l</i> -Tryptophane		<1	<1		
<i>dl</i> -Valine		<1	<1		

\* Average of nine experiments; range <0.4 to 2.6.

† Average of five experiments; range 0.6 to 1.5.

Finally, although the sodium salts of none of these acids formed detectable quantities of nicotinamide when heated with glutamic acid, the ammonium salts of several did; namely, aspartic,  $\alpha$ -ketoglutaric, maleic, oxalacetic, and malic acids. The reaction mixtures all gave relatively low yields of nicotinamide and the results are therefore somewhat obscured by the fact that the ammonium glutamate controls also formed small

amounts that were appreciably greater than the traces found with the sodium salt. However, these ammonium glutamate controls, which were always run at the same time, consistently showed considerably less nicotinamide formation than did the mixtures of ammonium glutamate with the acids mentioned above. No significant increase over the control occurred

TABLE III

*Formation of Nicotinamide Equivalent from Sodium and Ammonium Salts of Dibasic Acids Heated with Asparagine or Glutamic Acid*

Acid	Base used for neutralization	Nicotinamide produced in 48 hrs.						
		Alone	With glutamic acid			With asparagine		
			Average	No of experiments	Range	Average	No. of experiments	Range
			micro-moles per mole	micro-moles per mole	micromoles per mole	micro-moles per mole		micromoles per mole
Glutamic acid	NH <sub>3</sub>	3.5	3	3 - 4.2	3.9	2	260 - 390	
dl-Aspartic acid	NaOH		1.0	1		<1	1	
" "	NH <sub>3</sub>		13	2	12 - 13	1.1	2	1.0- 1.2
Fumaric acid	NaOH		1.8	1		5.5	1	
" "	NH <sub>3</sub>	<1	5	1		12	2	12 - 13
Glutaric acid	NaOH		1.1	1		9.7	2	8.4- 11
" "	NH <sub>3</sub>	<1	3.9	1		26	2	25 - 27
α-Ketoglutaric acid	NaOH					15	2	13 - 17
" "	NH <sub>3</sub>	<1	10	2	9.5-10.5	34	3	24 - 52
Maleic acid	NaOH		2	1		12	1	
" "	NH <sub>3</sub>	<1	9.5	2	9 - 10	22	2	21 - 24
l-Malic acid	NaOH		<1	1		4.8	1	
" "	NH <sub>3</sub>	<1	8.8	3	6 - 12	4.2	2	3.6- 4.8
Oxalacetic acid	NaOH		1.7	1		9	1	
Diethyloxalacetate*	NH <sub>3</sub>	<1	9	2	8.4- 9.6	11	2	11 - 11
Succinic acid	NaOH		<1	1		<1	1	
" "	NH <sub>3</sub>	<1	4.2	1		<1	1	

Values for nicotinamide formation that are believed to represent significant increases over the controls with either compound alone are given in bold-faced type.

\* This compound was added as the sodium salt. The reaction mixtures were neutralized at intervals with ammonia to prevent development of an unfavorable (1) acidity owing to hydrolysis of the ester. When tested in the absence of asparagine and glutamic acid 1 mole of NH<sub>4</sub>Cl was added.

with ammonium succinate, glutarate, or probably fumarate. Although the ammonium salts of α-ketoglutaric, maleic, oxalacetic, and malic acids were capable of replacing either glutamic acid or asparagine, none gave detectable activity when heated alone.

Additional experiments have indicated that, with many of the asparagine-

amino acid mixtures, considerably more nicotinamide activity may be produced by treating with hydrogen peroxide for 2 days at room temperature, then autoclaving for 15 minutes, than by the 48 hours heating at 100°, and under certain conditions even more than by heating for 10 days with aeration. Also, with this hydrogen peroxide treatment appreciable, although usually smaller, quantities are produced from many amino acids and from the ammonium salts of several dicarboxylic acids alone, in the absence of both asparagine and glutamic acid. Under suitable conditions over 20 micromoles per mole could be formed by the action of peroxide on asparagine, glutamic acid, citrulline, ammonium  $\alpha$ -ketoglutarate, or methionine, and smaller amounts from hydroxyproline and the ammonium salts of succinic, fumaric, or glutaric acid. With methionine and  $\alpha$ -ketoglutarate, there was evidence from the shape of the growth-concentration curves that nicotinic acid rather than the amide was formed. These results have proved even more difficult to reproduce quantitatively than have those involving heat alone and respond more markedly to the presence or absence of metal salts, the response varying with the compound. Therefore no detailed description of the experiments can be given.

#### DISCUSSION

The large number of amino acids and related compounds that form easily detectable quantities of nicotinamide activity on heating with asparagine unfortunately yield no clues as to the course of the originally discovered reaction between asparagine and glutamic acid. The only compound yet found, methionine, that is as active as the latter, could not in any one simple reaction even give a common intermediate. Among the amino acids, one generalization is obvious: all the terminal substituted 5-carbon amino acids, citrulline, ornithine, proline, arginine, and hydroxyproline, as well as glutamic acid, can take part in the reaction with asparagine, and these with the exception of arginine and of hydroxyproline, which has an additional substituent, are the most active of the compounds tested other than methionine. In view of the marked reactivity of the latter, it may be assumed that the terminal carbon atom of the 5-carbon amino acids, the 5th and 6th carbon atoms of lysine, and the  $\text{SCH}_3$  group of methionine are removed, possibly by oxidation, at some stage in the reactions leading to the active product. However, if this terminal oxidation is an early step, it cannot be one involving the formation of any of the 4-carbon non-nitrogenous, dibasic acids tested or of aspartic acid, since all of these are much less active than glutamic acid, methionine, proline, citrulline, or ornithine when heated with asparagine. The activity of tyrosine, phenylalanine, serine, and threonine with asparagine is difficult to explain on the basis of any possible relation to glutamic acid or its decomposition products. How-



ever, the activity of all four is quite low and may possibly be disregarded in a consideration of the reaction mechanism.

After it was found that oxygen is probably required for the reaction, it seemed possible that the first steps might involve an oxidation of one of the amino acids to a keto acid, followed by a condensation of the carbonyl group with the amino group of the other amino acid, as postulated for the Herbst reaction (5, 6). However, the relatively low activity of  $\alpha$ -ketoglutaric and oxalacetic acids would seem to preclude such reactions, at least as first steps. In fact the saturated glutaric acid is nearly as active when heated with asparagine as is  $\alpha$ -ketoglutaric acid, and aspartic acid is slightly more active than oxalacetic when heated with glutamic acid in the presence of ammonia.

The only effective substitute for asparagine yet found is another amide, glutamine (1), in contrast to the several different compounds that can replace glutamic acid, and, as previously discussed, the results obtained with glutamine are open to some doubt owing to the impurity of the sample tested. The experiments with the dibasic acids listed in Table III would seem to indicate that only some compound containing an amide group will be capable of fully replacing asparagine, since in all cases the sodium salts were completely inactive, whereas several of the ammonium salts, from which traces of amide might well be formed, showed small but distinct activity. It seems probable that the amide group has some essential function in the reaction other than merely that of leading to the formation of nicotinamide instead of the free acid. Otherwise, the latter should have been produced in amounts readily detectable by the method of assay used in the experiments in which replacements of amide or ammonium by sodium completely prevented the formation of the active product. The nature of the function of the amide group is still obscure.

#### SUMMARY

It is shown that the reaction between asparagine and glutamic acid that results in the formation of a nicotinamide-like substance is catalyzed by manganese and iron salts and promoted by aeration.

Certain amino acids and non-nitrogenous dibasic acids have been found capable of substituting for glutamic acid in the above reaction. In the order of decreasing activity they are methionine, proline, citrulline, ornithine,  $\alpha$ -ketoglutaric acid, glutaric acid, maleic acid, arginine, phenylalanine, hydroxyproline, fumaric acid, tyrosine, oxalacetic acid, lysine, serine, threonine, and malic acid.

The ammonium salts of a few dibasic acids, aspartic,  $\alpha$ -ketoglutaric, maleic, and malic, when heated with glutamic acid, produced small amounts of nicotinamide activity although their sodium salts were completely inactive.

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# STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

## XII. THE EFFECT OF *l*-ASCORBIC ACID ON THE HYPOPROTHROMBINEMIA INDUCED BY 3,3'-METHYLENEBIS(4-HYDROXYCOUMARIN) IN THE GUINEA PIG\*

By WILLIAM R. SULLIVAN, EDWARD O. GANGSTAD, AND KARL PAUL LINK

(From the Department of Biochemistry, Wisconsin Agricultural Experiment Station, University of Wisconsin, Madison)

(Received for publication, September 11, 1943)

The duration and extent of the hypoprothrombinemia induced by the anticoagulant and hemorrhagic agent 3,3'-methylenebis(4-hydroxycoumarin) (1) can be modified in certain rabbits by the administration of *l*-ascorbic acid (2) and in rats by substances (carvone, chloretone, etc.) that accelerate the synthesis of this vitamin (3). It therefore became desirable to determine whether similar responses could be observed in the guinea pig, since the ascorbic acid available to this species is subject to better experimental control.

Roderick and Schalk ((4) p. 31) had indicated that in some respects the lesions of the hemorrhagic sweet clover disease of cattle resemble those of scurvy. According to Macrae (5) Sir A. E. Wright held that scurvy eventuates in a defect of blood coagulability. Hess and Fish (6) and Presnell (7) reported that the clotting time of whole blood is prolonged in scurvy. However, little information appears to be available on the effect of *l*-ascorbic acid on the individual components of the blood-clotting mechanism except for platelets and calcium which remain unaffected by the vitamin deficiency. The present report therefore includes a study of the effect of *l*-ascorbic acid *per se* on the prothrombin level (or activity) in the plasma of normal and scorbutic guinea pigs.

### *Methods*

The method of estimating the prothrombin level (or activity) and the reasons for expressing the prothrombin responses by simply indicating the prothrombin time in seconds on whole plasma and 12.5 per cent plasma

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(1 part of plasma, 7 parts of saline solution) have been given in previous papers of this series ((8, 9) specifically pp. 942 and 950-953). The thromboplastin was prepared from guinea pig brain by the method used for rabbit brain thromboplastin (8). Blood samples were taken by heart puncture under light ether anesthesia. The 3,3'-methylenebis(4-hydroxycoumarin) was given orally in gelatin capsules, the ascorbic acid by pipette in freshly prepared aqueous solution. The scorbutogenic ration of Wolbach and Howe (10) was used as the basal ration. The 160 guinea pigs used in this study were young animals of both sexes, 4 to 6 months of age, ranging in weight from 260 to 600 gm. The number of guinea pigs used in the different experiments varied. More animals were always included in the scurvy groups than in the others because of the increased likelihood of death before the termination of the experimental period. Unless otherwise indicated, the results given represent an *average* value of groups of six or more guinea pigs, selected on the basis of uniformity in weight at the beginning of the experiment. The body weights of the guinea pigs were recorded throughout the course of each experiment, but no useful purpose would be served by including them.

#### EXPERIMENTAL

*Effect of Anticoagulant on Prothrombin Time of 12.5 Per Cent Plasma*—Twenty-five guinea pigs (average weight 460 gm.) maintained on the Wolbach and Howe ration, supplemented with 2 mg. of *L*-ascorbic acid daily, were fed 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin). Since a typical response curve for *whole* guinea pig plasma has already been published<sup>1</sup> (see (9) Fig. 2, p. 945), it will suffice to indicate the average prothrombin time of the 12.5 per cent plasma, along with the standard deviations at each point of sampling.

The prothrombin time of the normal 12.5 per cent plasma was 52 seconds ( $\pm 4.0$ ). The anticoagulant prolonged the time to 76 seconds ( $\pm 8.0$ ) in 24 hours and 85 seconds ( $\pm 13.0$ ) in 36 hours. By the 48th hour the time dropped to 69 seconds ( $\pm 10.0$ ), while approximately normal values were restored in 72 hours.

The response of individual guinea pigs to a standard dose of the anticoagulant is less reproducible than that of other species (rabbit, rat, or dog). Since the guinea pigs used in this work were by necessity young growing animals, the individual variation in response was most likely augmented by this factor. Our experience indicates that after an animal has reached full growth the response to the anticoagulant is subject to less daily variation.

<sup>1</sup> The guinea pigs used in this work were mature, the diet different, and the dosage of the anticoagulant was 10 mg.

*Effect of l-Ascorbic Acid Intake on Prothrombin Time of Guinea Pig Plasma*—The 82 guinea pigs (average weight 320 gm.) used in this experiment were first maintained on the scorbutogenic diet supplemented with raw cabbage for 2 weeks. During this interval the *normal* prothrombin times on whole plasma and 12.5 per cent plasma were obtained. The cabbage was then omitted and the following supplements were fed: Group A, no supplement; Group B, 0.5 mg. of *l*-ascorbic acid per day; Group C, 2 mg. of *l*-ascorbic acid per day; Group D, 25 mg. of *l*-ascorbic acid per day;

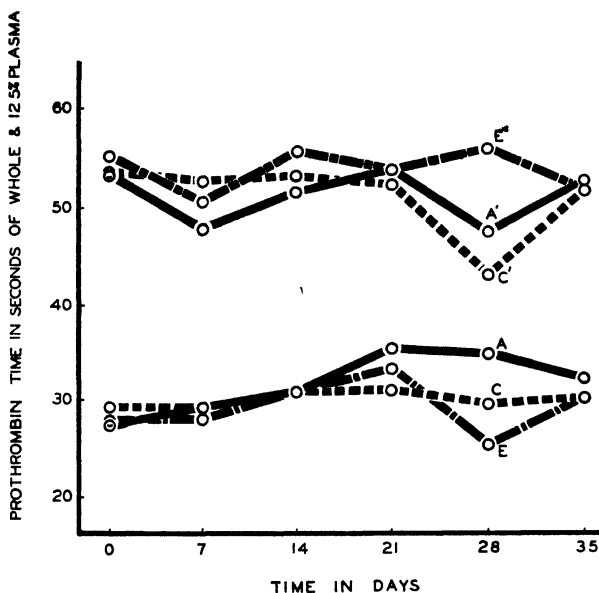


FIG. 1. The effect of *l*-ascorbic acid intake on the prothrombin time of guinea pig plasma. Curves A, C, and E represent whole plasma of guinea pigs receiving (Group A) no *l*-ascorbic acid, (Group C) 2 mg. of *l*-ascorbic acid per day, and (Group E) 40 to 50 gm. of raw cabbage per day. Curves A', C', and E' represent 12.5 per cent plasma from the same animals.

and Group E, 40 to 50 gm. of raw cabbage per day. Blood samples were taken at 7 day intervals.

The average values for Groups A, C, and E are plotted in Fig. 1. The only variation that is significantly outside the range of experimental error is observed in Curve A, where a definite increase from a normal value of 28 seconds to 36 seconds occurred in the whole plasma in the 3rd and 4th weeks. The slight drop in the prothrombin time in the 5th week is undoubtedly due to the fact that only the animals with a higher resistance to *l*-ascorbic acid depletion were still alive. The values for Group B

closely paralleled those for Group A, while those for Group D remained within the experimental error near the values for Groups C and E.

It is clear that total and partial depletion of *L*-ascorbic acid (Groups A and B) produced no change in the prothrombin time of the 12.5 per cent plasma, while a definite *increase* in clotting time was noted on the corresponding whole plasma. In contrast, adequate or high intakes of *L*-ascorbic acid did not affect the prothrombin time of either plasma concentration.

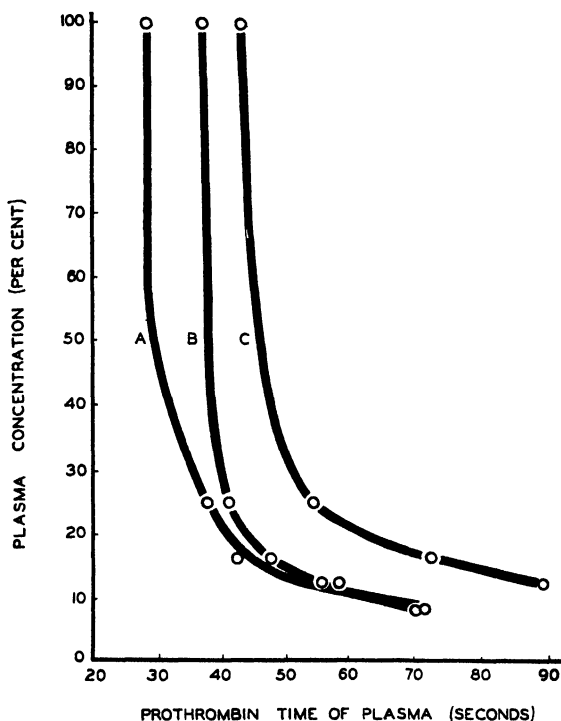


FIG. 2. The effect of dilution with saline solution on the clotting time of guinea pig plasma. Curve A represents plasma from normal guinea pigs (average values from ten animals), Curve B from a typical scorbutic animal, and Curve C a normal guinea pig 36 hours after administration of 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin).

The difference between the prothrombin times of normal and scorbutic whole plasmas disappears when they are diluted with 7 parts of saline solution (see Fig. 2). If the differences in the clotting times of the whole plasmas were due to variations in prothrombin level (or activity), Curve B of Fig. 2 would have remained to the right of and above Curve A, in a manner similar to Curve C (see also (8) Fig. 1, p. 8, and (9) Fig. 5, p. 951). Since it does not do so, the prolongation of the clotting time of *whole* scorbutic plasma cannot be ascribed to prothrombin depletion. Control

tests for antithrombins in the scorbutic plasmas were made by the method of Eagle (11) and were negative. This rules out the action of heparin or a like substance, and the action of other coagulation-inhibiting factors must be assumed (12). It should be indicated that we have observed this phenomenon repeatedly from causes other than vitamin C deficiency during the past 6 years. The same observation has been made on human plasma by Shapiro and coworkers (13). It is possible that in these cases a natural coagulation inhibitor is operating which is rendered inactive by dilution (12, 14). The observed increase in the clotting time of whole plasma from scorbutic guinea pigs correlates with the findings of Presnell (7) who reported that the coagulation time of guinea pig whole blood was increased 54 per cent in scurvy.

*Effect of l-Ascorbic Acid on Action of Anticoagulant in Non-Scorbutic Guinea Pigs*—The results from many trials will be reported in a highly condensed form. The control experiment with normal guinea pigs on the basal diet supplemented with 2 mg. of *l*-ascorbic acid showed that 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin) prolonged the prothrombin time of the 12.5 per cent plasma from 52 seconds ( $\pm 4.0$ ) to the maximum of 85 seconds ( $\pm 13.0$ ) in 36 hours, and that normal prothrombin values were restored within 72 hours. Single doses of *l*-ascorbic acid at levels as high as 100 mg. given either orally or intravenously along with 5 mg. of the anticoagulant did not materially alter the action of the anticoagulant. When 100 mg. of *l*-ascorbic acid were fed daily for 4 days prior to feeding 5 mg. of the anticoagulant, and also with the anticoagulant, the vitamin reduced the hypoprothrombinemia-inducing action significantly. The prothrombin time of the 12.5 per cent plasma at the 36th hour was 66 seconds, in contrast to the control figure of 85 seconds, when no *l*-ascorbic acid was given.

*Response of Scorbutic Guinea Pigs to Anticoagulant*—The effect of vitamin C deficiency on the response to a single dose of 3,3'-methylenebis(4-hydroxycoumarin) was studied on two uniform groups of young guinea pigs. Group I was fed only the basal scurvy diet, while Group II received a daily supplement of 40 to 50 gm. of fresh cabbage.<sup>2</sup> Blood samples were taken before 5 mg. of the anticoagulant were fed and 36 hours after the feeding. The prothrombin times of the plasma samples taken before

<sup>2</sup> It should be indicated that consideration was also given to the possibility that raw cabbage supplied other factors than vitamin C. When vitamin K (2-methyl-1,4-naphthoquinone) was given at levels from 5 to 50 mg. to guinea pigs maintained on the basal scurvy diet supplemented with 2.0 mg. of *l*-ascorbic acid, the response to 5.0 mg. of the anticoagulant was not affected. Furthermore guinea pigs maintained on the basal scurvy diet supplemented with 2.0 mg. of *l*-ascorbic acid did not show a vitamin K deficiency as reflected by the prothrombin times.



the anticoagulant was administered remained normal throughout the course of the experiment.

A striking difference in the response of the two groups was noted after 3 weeks of vitamin C depletion and this difference increased during the 4th and 5th weeks. Fig. 3 gives the prothrombin times of the plasmas 36 hours after the anticoagulant is fed. Curves A and A' are the prothrombin times of the 12.5 per cent and whole plasma respectively of Group I,

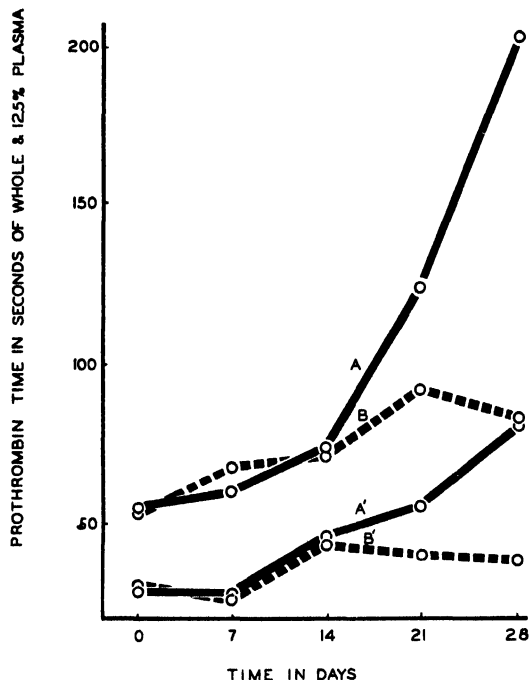


FIG. 3. The effect of vitamin C depletion on the response of guinea pigs to 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin). Curves A and A' represent average values for 12.5 per cent and whole plasmas respectively of the scorbutic guinea pigs 36 hours after the anticoagulant is fed. Curves B and B' are corresponding curves for guinea pigs protected against scurvy with fresh cabbage.

while Curves B and B' are the corresponding curves for Group II. Similar results were obtained with groups of six mature animals on the scurvy diet. The extent of the hypoprothrombinemia induced by the standard dose of the anticoagulant increased consistently as the vitamin C deficiency progressed.

*Recovery of Scorbutic Guinea Pigs from Effects of Single Dose of Anticoagulant*—The previous experiments indicate that the scorbutic state

affects the *extent* of the hypoprothrombinemia induced by the standard 5 mg. dose of 3,3'-methylenebis(4-hydroxycoumarin). The following results indicate that the *duration* of the hypoprothrombinemia is also drastically prolonged. It should be indicated that experimental difficulties were encountered in these trials, which could not be overcome. Blood samples cannot be taken at frequent intervals from scorbutic guinea pigs that have been given the anticoagulant. The guinea pigs become extremely liable to fatal hemorrhage following heart puncture. Indeed, in many instances, scorbutic animals that died a week after the last blood sample was drawn contained large pools of unclotted or partially clotted blood in the thoracic cavity. Since the variation in the prothrombin times between individuals was found to be very large, it becomes necessary to indicate the responses of individual but representative animals. A guinea pig on the basal scurvy diet for 9 weeks, supplemented with 0.5 mg. of *l*-ascorbic acid, responded to 5 mg. of the anticoagulant by an increase in the prothrombin time of the 12.5 per cent plasma from the normal of 52 seconds to 150 seconds in 36 hours. 7 days later the prothrombin time of the 12.5 per cent plasma was over 300 seconds.

A guinea pig that had received the basal scurvy diet and only 0.5 mg. of *l*-ascorbic acid for 10 weeks, and no supplement for the next 14 days, was fed 5 mg. of the anticoagulant. No prothrombin activity could be detected at the 36 hour, nor after 7 days, when death followed the second sampling. In a mature guinea pig, restricted for 4 weeks to the scurvy diet, 5 mg. of the anticoagulant prolonged the prothrombin time of the 12.5 per cent plasma to 87 seconds in 36 hours, and 7 days later the value was 89 seconds.

These responses indicate that in vitamin C-depleted guinea pigs the capacity to restore normal prothrombin levels *after* administration of the anticoagulant is markedly impaired. In sharp contrast, when a single dose of the anticoagulant is given to a normal guinea pig, as well as other species of animals (including man), the induced hypoprothrombinemia is quite transient, and is followed by rapid restoration of normal prothrombin levels (8, 9). The observation of Richards and Cortell (15) that in scorbutic guinea pigs the anticoagulant caused histological changes in the liver, characterized by fatty infiltration and degeneration, is of interest in this connection.

We have also observed (unpublished data) that vitamin C-depleted guinea pigs succumb earlier to the action of 3,3'-methylenebis(4-hydroxycoumarin) than normal ones. This has also been confirmed by Richards and Cortell (15). For example, the survival time of guinea pigs receiving 1.0 mg. of the anticoagulant per day could be extended from 15 to 20 days to over 60 days by the administration of 25 mg. of *l*-ascorbic acid per day.

The increased susceptibility to the prothrombin-reducing action of 3,3'-methylenebis(4-hydroxycoumarin) in scurvy, the delayed recovery in scurvy, and conversely the detectable decreased susceptibility to the anticoagulant in the presence of abundant *l*-ascorbic acid suggest that adequate vitamin C levels are necessary for rapid prothrombin synthesis. In Paper X of this series (3) it was shown that substances that stimulate the synthesis of vitamin C in the rat affect the mechanism by which the hypoprothrombinemia induced by 3,3'-methylenebis(4-hydroxycoumarin) is counteracted.

#### SUMMARY

1. The responses of non-scorbutic and scorbutic guinea pigs to the oral administration of 5.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) are given. The extent and duration of the hypoprothrombinemia induced by the anticoagulant are markedly increased in scurvy.

2. Total and partial depletion of vitamin C in the guinea pig produced no change in the prothrombin time of 12.5 per cent plasma. Adequate or high intakes of *l*-ascorbic acid did not affect the prothrombin time of 12.5 per cent plasma.

3. In guinea pig scurvy the clotting time of the *whole* plasma is increased slightly. Evidence is given that this is not due to a change in the prothrombin level (or activity).

4. Single 100 mg. doses of *l*-ascorbic acid did not protect non-scorbutic guinea pigs from the hypoprothrombinemia induced by 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin). Repeated 100 mg. doses of the vitamin exert a detectable protective effect.

Our thanks are due to Mr. Harland D. Wycoff for help with the bioassays. We are also indebted to Dr. Carl A. Baumann for his counsel and valuable suggestions. (K. P. L.)

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## MERCAPTURIC ACIDS

### III. THE CONVERSION OF BENZENE TO PHENYLMERCAPTURIC ACID IN THE RAT

BY S. H. ZBARSKY AND LESLIE YOUNG

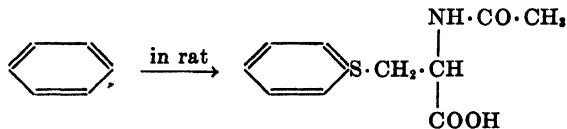
(From the Department of Biochemistry, University of Toronto, Toronto, Canada)

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During the latter part of the nineteenth century Baumann and his co-workers (1, 2) and Jaffe (3) showed that certain monohalogen-substituted benzenes are converted to mercapturic acids in the dog, and that these derivatives can be isolated from the urine of dosed animals. No other mercapturic acid was isolated from the urine of animals receiving a foreign organic compound until Bourne and Young (4) obtained *l*- $\alpha$ -naphthylmercapturic acid from the urine of rabbits dosed with naphthalene and thus established that mercapturic acid formation can occur *in vivo* following the administration of an *unsubstituted* aromatic hydrocarbon. Another example of this type of metabolic reaction was reported later by Boyland and Levi (5), who showed that 1-anthrylmercapturic acid is excreted in the urine of rats and rabbits dosed with anthracene.

The fact that mercapturic acid formation follows the administration of naphthalene, anthracene, or a monohalogen-substituted benzene led to speculation concerning the possibility that benzene undergoes a similar change in the body. The belief that such a change occurs was supported by experimental evidence from several sources, although in no case was the isolation of phenylmercapturic acid reported. Callow and Hele (6) found that the administration of benzene to dogs was followed by an increase in the neutral sulfur content of the urine and a similar observation was made by Stekol (7). Drummond and Finar (8) obtained evidence that phenylmercapturic acid was excreted in the urine of rabbits dosed with benzene, but they were unable to isolate the compound from the urine.

The synthesis of *l*-phenylmercapturic acid *in vitro* and the isolation of this compound from the urine of rats which had ingested phenyl-*l*-cysteine were described in earlier papers of the present series (9, 10). The experiments described below establish that excretion of phenylmercapturic acid follows the administration of benzene to the rat.



The isolated compound was identified by analysis and by comparison of its properties with those of synthetic *l*-phenylmercapturic acid prepared as described in Paper I of the present series (9).

#### EXPERIMENTAL

In all experiments the animals used were male white rats each weighing from 200 to 300 gm. They were housed in metabolism cages which permitted the collection of urine free from feces. They were fed in a separate cage twice daily for periods of 1 hour each and the urine excreted during these periods was not collected. The animals had access to drinking water at all times. In some experiments they received a diet (Diet A) composed of ground corn 50, dried milk 25, linseed meal 15, casein 3.5, alfalfa meal 1.5, calcium carbonate 0.5, sodium chloride 0.5, yeast powder 2, and cod liver oil 2 parts. In other experiments the diet (Diet B) consisted of Master Fox Breeding Ration (Toronto Elevators Ltd.) supplemented with fresh milk daily and whole wheat bread twice a week.

The benzene was administered to the rats by stomach tube while they were under ether anesthesia. In some experiments a solution of 40 per cent of benzene (by volume) in corn oil was given and in others the benzene was administered without a diluent. The urine was collected daily throughout the period during which the animals were dosed and for 2 days afterwards. Because phenylmercapturic acid tends to decompose in an alkaline medium, the collected urine was kept just acid to litmus by the daily addition of hydrochloric acid and stored in the refrigerator.

Although the methods employed to isolate phenylmercapturic acid from the urine of rats dosed with benzene differed in detail from experiment to experiment, they were all based on that used to obtain phenylmercapturic acid from the urine of rats which had ingested phenyl-*l*-cysteine (10). The urine was made acid to Congo red by the addition of hydrochloric acid, allowed to stand overnight in the refrigerator, and extracted three times with portions of chloroform each equal in volume to that of the acidified urine. Phenylmercapturic acid was isolated by fractionation of the combined chloroform extracts. The isolation of phenylmercapturic acid from the urine of rats dosed with benzene was more difficult than the isolation of this compound from the urine of rats to which phenyl-*l*-cysteine had been administered. This arose in part from the fact that the concentration of phenylmercapturic acid in the urine was much lower when the rats were dosed with benzene than when they received phenyl-*l*-cysteine. An additional difficulty was introduced by the fact that the urine of rats dosed repeatedly with benzene was darkly colored and the pigmented substances tended to be extracted from the acidified urine by chloroform. The main problem in the isolation process was the separation of phenylmercapturic

acid from the dark, oily products which were obtained by fractionation of the chloroform extract. It was observed, however, that certain of these products contained crystals after they had been left in the refrigerator for periods up to 2 months. It was found possible to free these crystals from the contaminating oily material by washing them with small amounts of cold ethanol. The crystals, after being subjected to further purification, were found to consist of phenylmercapturic acid.

Four experiments were performed in which phenylmercapturic acid was isolated from the urine of rats dosed with benzene. Details concerning these experiments are given below and the isolation procedure followed in one of them (Experiment IV) is shown.

*Experiment I*—Twelve rats were fed Diet A and each received by stomach tube 0.25 ml. of a 40 per cent solution (by volume) of benzene in corn oil (Mazola) daily for 8 days. The animals were rested for a week and were then dosed daily for a further period of 7 days. The total weight of benzene administered to these animals was 15.8 gm. and from the urine 0.177 gm. of phenylmercapturic acid was isolated. The identity of the compound obtained from the urine was established by the following observations.

The isolated compound melted<sup>1</sup> at 142° and when it was mixed with synthetic *l*-phenylmercapturic acid, m.p. 142°, the melting point was not depressed. It yielded the following results when analyzed.

$C_{11}H_{13}O_2NS$ .	Calculated.	C 55.21, H 5.47, N 5.86, S 13.39
	Found.	" 55.25, " 5.58, " 6.01, " 13.25

The determination of its equivalent weight by titration with 0.02 *N* sodium hydroxide solution yielded 238, 242. The calculated equivalent weight of phenylmercapturic acid is 239.

The specific rotation of a 1 per cent solution of the isolated compound in ethanol was  $[\alpha]_D^{20} = -23^\circ$ . Under these conditions the specific rotation of synthetic phenylmercapturic acid was found to be  $-23^\circ$  (9).

*Experiment II*—Twenty-four rats were fed Diet B and each received by stomach tube 0.25 ml. of a 40 per cent solution (by volume) of benzene in corn oil daily for 3 days. The total weight of benzene administered was 6.3 gm. The phenylmercapturic acid isolated from the urine weighed 0.032 gm. It melted at 142° and when it was mixed with synthetic phenylmercapturic acid the melting point was not depressed.

$C_{11}H_{13}O_2NS$ .	Calculated.	C 55.21, H 5.47, N 5.86
	Found.	" 55.57, " 5.76, " 5.93

*Experiment III*—Eleven rats were fed Diet A and each received by stomach tube 0.1 ml. of benzene daily for 6 days. The total weight of

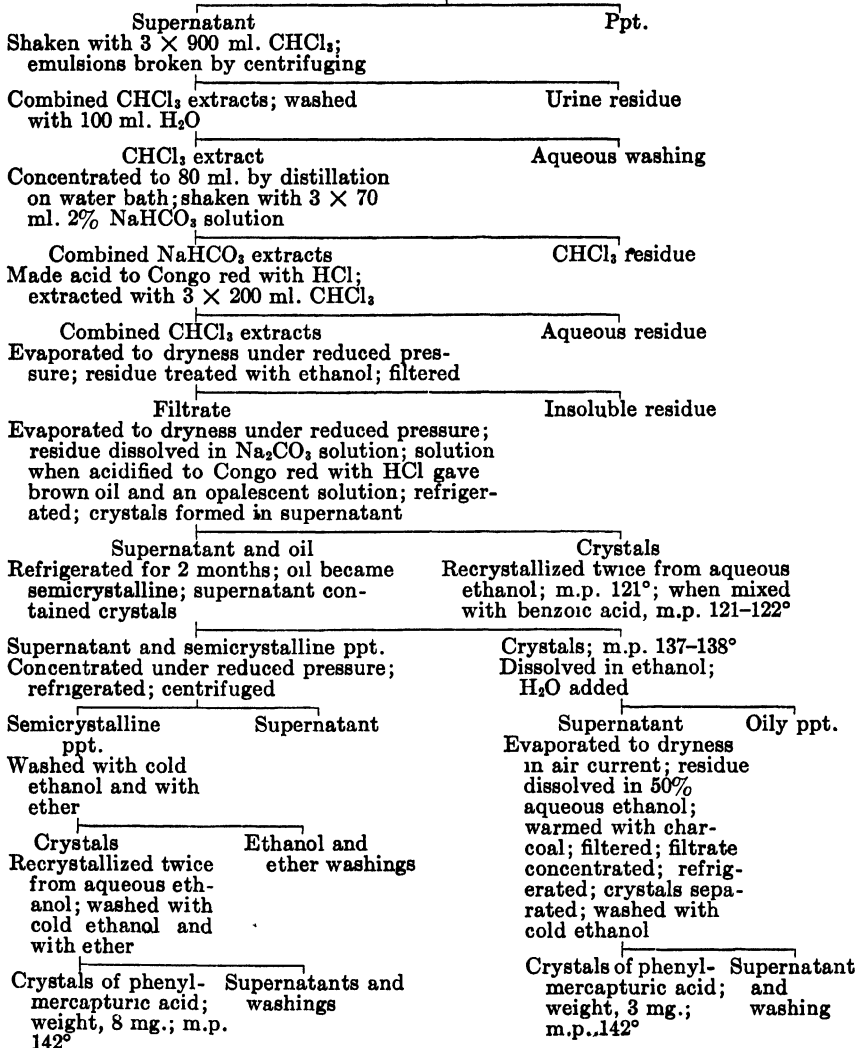
<sup>1</sup> All melting points recorded herein are uncorrected.



benzene administered was 5.8 gm. The weight of phenylmercapturic acid isolated from the urine was 0.020 gm. The isolated compound melted at 141–142° and a mixture of this material and synthetic phenylmercapturic

*Isolation of Phenylmercapturic Acid from Urine of Rats Dosed with Benzene  
(Experiment IV)*

Urine (900 ml.) from 12 rats each dosed with 0.1 ml. benzene daily for 6 days (total benzene intake, 6.3 gm.). Urine made acid to Congo red with HCl; refrigerated overnight; centrifuged



acid melted at 141–142°. The isolated compound yielded the following results when analyzed.

$C_{11}H_{13}O_2NS$ .	Calculated.	C 55.21, H 5.47, N 5.86
	Found.	" 55.29, " 5.67, " 5.84

*Experiment IV*—Twelve rats were fed Diet B and each received by stomach tube 0.1 ml. of benzene daily for 6 days. The total weight of benzene administered was 6.3 gm. and the phenylmercapturic acid isolated from the urine weighed 0.011 gm. The isolated material melted at 142° and when it was mixed with synthetic phenylmercapturic acid the melting point was not depressed.

$C_{11}H_{13}O_2NS$ .	Calculated.	C 55.21, H 5.47, N 5.86
	Found.	" 55.44, " 5.66, " 5.72

Because corn oil was used as a diluent for the benzene in Experiments I and II, a control experiment was performed in which 0.15 ml. of corn oil was administered daily by stomach tube to each of twelve rats for 6 days. No phenylmercapturic acid was obtained when the urine of these animals was treated by the method applied to the urine of rats dosed with benzene.

#### DISCUSSION

The metabolism of benzene has been the subject of many investigations and these have revealed that this hydrocarbon is converted to a variety of compounds in the animal body. As long ago as 1867 Schultzen and Naunyn (11) found that benzene is converted to phenol in man and the dog and later work showed that the latter compound is excreted in conjugation with sulfuric acid (12) and with glucuronic acid (13). It has also been established that the oxidation of benzene *in vivo* gives rise to the formation of catechol (14), quinol (14), and muconic acid (8, 15, 16). The conversion of benzene to phenylmercapturic acid, although recognized as a possible metabolic reaction, has not hitherto been supported by evidence based on the isolation of this compound. Phenylmercapturic acid has now been obtained from the urine of rats dosed with benzene and as a result of this finding benzene takes its place with other aromatic hydrocarbons (naphthalene, anthracene), the administration of which is followed by the excretion of mercapturic acids.

Although the present work throws no light on the mechanism of mercapturic acid formation *in vivo*, it is noteworthy that phenylmercapturic acid excretion follows the ingestion of phenyl-*l*-cysteine by the rat (10). Formation of phenylmercapturic acid in the rat appears to occur more readily from phenyl-*l*-cysteine than from benzene. In experiments (10) in which rats ingested phenyl-*l*-cysteine, as much as 38 per cent of the

compound was recovered from the urine as phenylmercapturic acid, whereas in the experiments described herein the amounts of phenylmercapturic acid isolated from the urine were small. When a solution of benzene in corn oil was given to rats, the amounts of the acid which were isolated from the urine corresponded to 0.37 per cent (Experiment I) and 0.17 per cent (Experiment II) of the benzene administered. When benzene was given without a diluent, the amounts of phenylmercapturic acid obtained from the urine corresponded to 0.11 per cent (Experiment III) and 0.06 per cent (Experiment IV) of the benzene administered. Even if allowance is made for the possibility that losses in the isolation of phenylmercapturic acid from the urine of rats dosed with benzene contributed to the small amounts of the compound obtained, it would nevertheless seem probable that in a quantitative sense phenylmercapturic acid is an unimportant metabolic product of benzene.

#### SUMMARY

Phenylmercapturic acid has been isolated from urine excreted by rats to which benzene had been administered by stomach tube. The isolated compound has been identified by analysis and by comparison of its properties with those of synthetic phenylmercapturic acid.

One of us (S. H. Z.) is indebted to the Banting Research Foundation for a personal grant.

The microanalyses reported herein were kindly performed by Mr. Michael Edson.

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# EFFICIENCY OF AEROBIC PHOSPHORYLATION IN CELL-FREE HEART EXTRACTS\*

By SEVERO OCHOA

*(From the Department of Medicine, New York University College of Medicine, and the Medical Service of the Psychiatric Division, Bellevue Hospital, New York)*

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When pyruvic acid is oxidized by tissue extracts in the presence of inorganic phosphate and of phosphate acceptors such as hexose monophosphate, glucose, or creatine, there is a coupled phosphorylation of the acceptor proportional to the oxygen consumption. Under suitable conditions 2 molecules of phosphate may be taken up for every atom of oxygen consumed<sup>1</sup> (2, 3). The ratio, atoms of P to atoms of oxygen, hereafter referred to as the P:O ratio, is a measure of the efficiency of conversion of oxidation energy to phosphate bond energy (4).

Various lines of evidence indicate that the coupled phosphorylation is the result of (1) phosphorylation of adenylic acid to adenosine polyphosphate coupled with oxidation, and (2) transfer of phosphate from adenosine polyphosphate to the phosphate acceptor. Through the change adenylic acid  $\rightleftharpoons$  adenosine triphosphate the adenylic acid system acts as a catalytic carrier of inorganic phosphate to the acceptor.<sup>2</sup>

Such a reaction mechanism is supported by the following facts. (a) A catalytic amount of adenylic acid (or adenosine polyphosphate) is essential for aerobic phosphorylation (3); (b) with adenylic acid as the only phosphate acceptor there is aerobic formation of acid-labile phosphate (2, 6); (c) the same tissue preparations in which aerobic phosphorylation occurs contain enzymes that catalyze reactions of type (2) (*cf.* (3)); and (d) aerobic

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<sup>1</sup> Belitzer and Tsibakowa (2) obtained higher values when they used for the calculation the excess oxygen uptake which, in their experiments, was caused by addition of the phosphate acceptor, but this calculation is objectionable.

<sup>2</sup> As is well known reactions involving the adenylic acid system may take place over either or both of the steps, adenosine triphosphate  $\rightleftharpoons$  adenosine diphosphate, adenosine diphosphate  $\rightleftharpoons$  adenylic acid. In tissues that contain myokinase (5) the three nucleotides form an equilibrium according to the equation, 2 adenosine diphosphate  $\rightleftharpoons$  adenosine triphosphate + adenylic acid.

phosphorylation of glucose in certain muscle extracts is negligible unless hexokinase<sup>3</sup> is added (8).

Since the enzyme adenosinetriphosphatase, which hydrolyzes adenosine triphosphate to adenylic acid and inorganic phosphate, is present in all tissue preparations in which aerobic phosphorylation can be studied, it must compete with the enzymes catalyzing the transfer of phosphate from adenosine triphosphate to phosphate acceptors. It follows that the value of 2 previously obtained for the P:O ratio of pyruvate oxidation is probably too low, a conclusion that is supported by evidence to be presented in this paper. The P:O ratio of pyruvate oxidation has now been redetermined by means of an indirect method and has been found to have a value of 3.

#### EXPERIMENTAL

##### *Methods*

*General*—Cat heart extracts were used throughout this work. The ventricles were freed from fat and connective tissue, chilled in ice, and minced as finely as possible with scissors. The mince was thoroughly ground in an ice-cold mortar with sand and 2 volumes of either 0.9 per cent potassium chloride or 0.1 M potassium phosphate buffer, pH 7.7, at 0°. The resulting paste was centrifuged for 2 to 3 minutes at about 2000 R.P.M., and the turbid supernatant was dialyzed for 2 to 3 hours in thin collodion membranes against 3 to 4 liters of either 0.4 per cent potassium chloride (KCl extracts) or 0.05 M potassium phosphate buffer, pH 7.7 (phosphate extracts), with vigorous outside stirring, at 1–2°. Reaction mixtures were prepared by making up 1.0 cc. portions of dialyzed extract to 1.5 to 1.75 cc. with various additions.

*Analytical*—The experimental samples were deproteinized with trichloroacetic acid and analyses were performed on aliquots of the filtrates. Orthophosphate was determined by the method of Fiske and Subbarow as modified by Lohmann and Jendrassik (9). Phosphocreatine was determined by the method of Lohmann (10). Fructose diphosphate was determined as fructose by Roe's method (11) with the exception that the period of heating was prolonged to 15 minutes; under these conditions fructose diphosphate gives 77 per cent of the color given by fructose. Lactic acid was determined by the method of Wendel (12), and pyruvic acid by the hydrazone method (13). A Summerson photoelectric colorimeter was used for all colorimetric estimations.

*Preparative*—Freshly made up solutions of crystalline sodium pyruvate were used as in previous work (3). Phosphocreatine was prepared syn-

<sup>3</sup> Hexokinase (7) catalyzes the transfer of phosphate from adenosine triphosphate to glucose, forming glucose-6-phosphate.

thetically by the method of Zeile and Fawaz (14). Fructose diphosphate was prepared after the method of Neuberg (15). Cozymase was prepared from bakers' yeast (Fleischmann)<sup>4</sup> by the method of Williamson and Green (16) with some modifications; assuming a water content of 8 per cent for the desiccator-dry product (17) its purity was about 90 per cent as judged from the P content and from reduction with platinum-hydrogen and with hydrosulfite. Adenosine triphosphate was prepared from rabbit muscle by the method of Lohmann (18) as modified by Cori,<sup>5</sup> and adenylic acid by alkaline hydrolysis of adenosine triphosphate after Lohmann (18).

All acids (except cozymase) were used as the sodium salts.

*Linkage of Pyruvate Oxidation with Phosphorylation of Adenylic Acid*—As mentioned above an increase of acid-labile phosphate has been reported in respiring tissue preparations after addition of adenylic acid (2, 6), suggesting a formation of adenosine polyphosphate. For the present work it was essential to reinforce this evidence for the coupling of oxidation with phosphorylation of adenylic acid by actual isolation of the adenosine polyphosphate formed.

The experimental samples (volume 1.5 cc.) were incubated in each of five Warburg vessels. Vessel 1, a control, contained glucose as phosphate acceptor and a catalytic amount of adenylic acid. Vessels 2 to 5 each contained adenylic acid as the only phosphate acceptor. Sodium fluoride was added to all samples in order to decrease the activity of the adenosinetriphosphatase (19). 0.5 cc. aliquots were removed from Vessels 1 and 2 at the end of the incubation period, for estimation of inorganic and acid-labile phosphate. The same values were secured at zero time from a sample containing no phosphate acceptor. The results are illustrated in Table I. The remainder of the sample in Vessel 2 together with the contents of Vessels 3 to 5, or a total of 5.5 cc. of reaction mixture containing 1.76 mg. of acid-labile P, was deproteinized with trichloroacetic acid and adenosine polyphosphate was isolated according to the usual procedure (18) through the barium and mercury salts and reversion to the barium salt. The final product contained 0.84 mg. of acid-labile P (yield 48 per cent). The molar ratio of acid-labile P to acid-stable P to pentose<sup>6</sup> was 1.02:1.00:0.96, indicating that the isolated substance was adenosine diphosphate. It is probable that adenosine triphosphate was formed and that the phosphatase split off more readily the third phosphate residue. Adenosine diphosphate could also have been formed from adenosine triphosphate and adenylic acid by the action of myokinase (5).

<sup>4</sup> I am greatly indebted to Dr. Charles N. Frey, The Fleischmann Laboratories, New York, for a generous supply of yeast.

<sup>5</sup> Cori, C. F., personal communication.

<sup>6</sup> Determined by the method of Mejbaum (20) with adenylic acid as standard.

**Adenosinetriphosphatase Activity**—In order to know whether the adenosinetriphosphatase in heart extracts can interfere with aerobic phosphorylation and the approximate extent of such interference, one has to study the hydrolytic action of the extracts on adenosine triphosphate under the same conditions of concentration and pH as in the aerobic experiments (*cf.* Tables V and VI). Two experiments of this type are illustrated in Table II. The minced heart was washed with 5 volumes of ice-cold 0.9 per cent potassium chloride before extraction. The reaction mixtures, in test-tubes, were shaken in a thermostat; at specified intervals the contents of each tube were deproteinized with trichloroacetic acid, and the filtrates analyzed for inorganic and acid-labile (adenosine triphosphate) P. As the samples were incubated in air, cyanide was added in order to prevent aerobic regeneration of adenosine triphosphate.

TABLE I

*Aerobic Phosphorylation of Adenylic Acid*

All samples contained 1.0 cc. of dialyzed phosphate extract, 0.005 M  $MgCl_2$ , 0.036 M pyruvate, 0.001 M succinate, and 0.025 M NaF. Temperature 37°. 100 per cent oxygen in the gas phase.

Additions		Incubation time	Oxygen uptake	Inorganic P	Acid-labile P*	P esterified	P esterified appearing as acid-labile P
Adenylic acid	Glucose						
$m \times 10^{-3}$	$m \times 10^{-3}$	min	c mm	mg	mg	mg.	per cent
		0		1.77	0.03		
3.7†	37	20	490	0.08	0.57	1.69	32
15.0‡		20	497§	1.29	0.50	0.48	98

\* Liberated by 10 minutes hydrolysis in 1.0 N  $H_2SO_4$  at 100°.

† 0.17 mg. of P.

‡ 0.7 mg. of P.

§ Average of Vessels 2 to 5 (see text).

Table II shows that the adenosinetriphosphatase activity of the extracts is very high. This activity was only partially inhibited (about 50 per cent) by 0.025 M sodium fluoride, higher concentrations being no more effective, but even under these conditions almost half of the adenosine triphosphate added was hydrolyzed in the first 5 minutes. The inhibitory effect of fluoride may be due to precipitation of calcium ions which are necessary for the action of adenosinetriphosphatase (21).

**Hexokinase**—Besides hexokinase tissue extracts contain phosphohexoisomerase (22), that establishes an equilibrium between glucose-6-phosphate and fructose-6-phosphate, and an enzyme that catalyzes the transfer of phosphate from adenosine triphosphate to the keto ester forming fructose-1,6-diphosphate (23) so that glucose is phosphorylated to fructose diphosphate.

The general procedure for the experiments of Table III was the same as in the previous section. It is apparent (Table III) that adenosinetriphosphatase interfered with the phosphorylation of sugar by adenosine triphosphate. Without fluoride all of the added adenosine triphosphate was broken down, 70 to 75 per cent of its acid-labile phosphate appearing as

TABLE II  
*Adenosinetriphosphatase*

All samples contained 1.0 cc. of dialyzed KCl extract, 0.005 M  $MgCl_2$ , 0.001 M adenosine triphosphate (0.1 mg. of acid-labile P), 0.03 M borate-KCl buffer (pH 7.7), and 0.006 M KCN. Incubation in air at 37°.

Experiment No.	Incubation time	Hydrolysis of adenosine triphosphate	
		Without NaF	With 0.025 M NaF
	min.	per cent	per cent
1	5	88	45
	10	90	56
	20	100	58
2	5	74	44
	10	86	54
	20	94	58

TABLE III  
*Hexokinase Reaction*

All samples contained 1.0 cc. of dialyzed KCl extract, 0.005 M  $MgCl_2$ , 0.016 (or 0.008) M glucose, 0.006 M adenosine triphosphate (0.6 mg. of acid-labile P), 0.02 M borate-KCl buffer (pH 7.7), and 0.005 M KCN. Incubation in air at 37° for 15 minutes.

Experiment No	NaF	$\Delta$ inorganic P (a)	$\Delta$ acid-labile P* (b)	(b) - (a)	Hexose diphosphate formed†
	$M \times 10^{-3}$	mg	mg	mg. P	mg. P
1		+0.45	-0.57	0.12	0.09
	25‡	+0.09	-0.31	0.22	0.16
2		+0.47	-0.61	0.14	0.09
	25‡	+0.09	-0.35	0.26	0.20

\* Liberated by 7 minutes hydrolysis in 1.0 N HCl at 100°.

† Calculated from fructose estimation.

‡ Averages of two samples.

orthophosphate while the remainder was transferred to glucose. In the presence of fluoride only about half of the adenosine triphosphate was broken down but of this only 25 to 30 per cent was hydrolyzed, the remainder of the acid-labile phosphate being transferred to the sugar. The net effect of fluoride was to double the amount of sugar phosphorylated



during incubation. However, even with fluoride the efficiency of the phosphate transfer reaction was only about three-fourths of what it should have been in the absence of adenosinetriphosphatase.

*Transfer of Phosphate from Phosphocreatine to Glucose*—Here phosphocreatine is the donor of phosphate to adenylic acid (24) forming adenosine triphosphate which passes its acid-labile phosphate over to the sugar. The adenylic acid system acts in a catalytic manner as a carrier of phosphate between phosphocreatine and glucose just as it acts catalytically in aerobic phosphorylation as a carrier of inorganic phosphate to the phosphate acceptor. If sufficiently active, adenosinetriphosphatase will interfere with the phosphorylation of the acceptor to a similar extent in both cases.

The experimental procedure was analogous to that of the two previous sections except that washing of the minced heart before extraction was

TABLE IV  
*Transfer of Phosphate from Phosphocreatine to Glucose*

All samples contained 1.0 cc. of dialyzed KCl extract, 0.005 M  $MgCl_2$ , 0.001 M adenosine triphosphate (0.1 mg. of acid-labile P), 0.024 M glucose, 0.03 M phosphocreatine (1.6 mg. of P), 0.03 M borate-KCl buffer (pH 7.7), and 0.006 M KCN. Incubation in air at 37° (All values are averages of two samples)

Experiment No	Incubation time	NaF	$\Delta$ phosphocreatine	$\Delta$ inorganic phosphate	$\Delta$ hexose diphosphate*
	min.	$M \times 10^{-3}$	mg P	mg P	mg P
1	15		-1.60	+1.23	+0.38
	15	25	-1.60	+0.89	+0.66
2	25		-1.63	+1.40	+0.30
	25	25	-1.63	+1.00	+0.64

\* Calculated from fructose estimation.

omitted because it removed a large part of the soluble enzymes that catalyze the reaction between phosphocreatine and adenylic acid. The whole of the phosphocreatine was broken down during the incubation period irrespective of the absence or presence of fluoride (Table IV), but whereas in the former case only about 20 per cent of its phosphate was transferred to glucose, twice as much was transferred in the presence of fluoride; the remainder appeared in all cases as inorganic phosphate. This is in agreement with previous results on brain dispersions (3) in which phosphopyruvic acid was used as phosphate donor in an otherwise identical system.

*Indirect Measurement of Efficiency of Aerobic Phosphorylation*—The above evidence suggests that fluoride increases the efficiency of transphosphorylations owing mainly to its partial inhibitory action on adenosinetriphosphatase. This poison increases the efficiency of aerobic phosphorylation

(2, 3, 25, 26), as it would now seem, for the same reason. Its effect on the aerobic phosphorylation of glucose in heart extracts is illustrated in Table V. Oxidation of pyruvate was inhibited by about 40 per cent, whereas phosphorylation of the sugar remained practically unchanged, so that the P:O ratios were correspondingly increased. Since the respiratory quotients remained unchanged in the presence of fluoride, it would seem that this poison merely depresses the oxidation of pyruvate without producing a qualitative change.

The experiments of the foregoing sections indicate that, even when fluoride is used, the directly measured P:O ratios of aerobic phosphorylation must be below their true value because part of the adenosine triphosphate formed by the oxidative phosphorylation of adenylic acid will be hydrolyzed by adenosinetriphosphatase and fail to phosphorylate the ac-

TABLE V  
*Effect of Fluoride on Aerobic Phosphorylation*

All samples contained 1.0 cc. of dialyzed phosphate extract, 0.005 M  $\text{MgCl}_2$ , 0.001 M adenosine triphosphate (0.1 mg. of acid-labile P), 0.036 (or 0.018) M pyruvate, 0.001 M succinate, and 0.037 M glucose. Temperature 37°. 100 per cent oxygen in the gas phase.

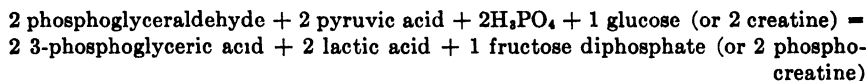
Experiment No	Incubation time	NaF	R.Q.	Oxygen uptake	Phosphate esterified	P:O ratio
	<i>min</i>	<i>M × 10<sup>-3</sup></i>		<i>microatoms</i>	<i>microatoms P</i>	
1	21		1.10	40.4	40.0	0.99
	21	25	1.17	27.2	39.0	1.44
2	17			40.7	40.3	0.99
	17	25		26.7	39.4	1.48
3	20		1.17	38.8	43.0	1.11
	20	25	1.08	26.4	40.0	1.52

ceptor. To the effect of adenosinetriphosphatase may be added some dephosphorylation of the phosphorylated acceptor or of intermediary carboxyl-phosphate bonds. From the above results it would appear that the P:O ratios determined directly must fall between 25 and 60 per cent short of the true value.

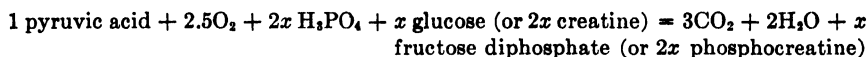
It is possible to obtain a more accurate estimate of the true P:O ratio of pyruvate oxidation by comparing the respective phosphorylations caused by this reaction and by the anaerobic dismutation between phosphoglyceraldehyde and pyruvate, if such a comparison is carried out in parallel experiments with the same enzyme preparation and under conditions that are identical as far as possible. The reaction mechanism is similar in both cases, inorganic phosphate being taken up by a phosphate acceptor over the the adenylic acid system through coupling with dehydrogenation (4, 27-29).

In the absence of adenosinetriphosphatase the dismutation causes the uptake of 1 molecule of inorganic phosphate for every molecule of phosphoglyceraldehyde dehydrogenated (27-29). Hence, irrespective of the actual P:O values obtained in any particular experiment, the ratio of aerobic to anaerobic phosphorylation, when referred to the same amount of hydrogen mobilized,<sup>7</sup> will be a fairly accurate measure of the true P:O value of the former.

In the experiments of Table VI the over-all anaerobic reaction was



and the over-all aerobic reaction was



The extracts used in this work failed to catalyze the dismutation unless cozymase was added. In all cases a catalytic amount of adenylic acid or adenosine triphosphate must be present.

Fructose diphosphate was used as the source of phosphoglyceraldehyde for the dismutation, since the extracts contain aldolase and isomerase (30). Fluoride was present throughout in order not only to depress adenosinetriphosphatase activity but also to stop further reaction of the phosphoglyceric acid formed in the dismutation. The volume of the experimental samples was 1.5 cc. Phosphoglycerate was determined as the difference between the orthophosphate liberated by ashing and by 180 minutes hydrolysis in 1.0 N HCl at 100° respectively. In the experiments with creatine as phosphate acceptor the assumption that an increase of that difference is exclusively due to the formation of phosphoglycerate is justified because there is no other difficultly hydrolyzable phosphate ester formed under those conditions; this may not be the case when glucose is the phosphate acceptor, because a little hexose monophosphate may accumulate. For this reason no estimations of phosphoglycerate were carried out in the glucose series.<sup>8</sup>

<sup>7</sup> For the present purpose 1 atom of oxygen consumed in respiration is equivalent to 1 molecule of phosphoglyceraldehyde oxidized to phosphoglyceric acid in the dismutation. In each case 2 hydrogen atoms are removed from the substrate; they reduce 1 atom of oxygen to water in the former case and 1 molecule of pyruvic acid to lactic acid in the latter. Both the number of atoms of oxygen consumed in one case and the number of molecules of substrate undergoing dismutation in the other are referred to in Table VI as "Equivalent oxygen reduced," and P:O ratios are expressed in identical units in both cases.

<sup>8</sup> In two experiments the increase of difficultly hydrolyzable ester was actually higher than could be accounted for by formation of phosphoglycerate.

In the anaerobic experiments both the pyruvate and fructose diphosphate were tipped in from the side bulb of Warburg vessels after temperature equilibration (usually 5 minutes). In the aerobic experiments, owing to the fact that the enzyme system is rather unstable in the absence of substrate, pyruvate was placed in the main space of the vessels. All vessels were

TABLE VI  
*Efficiency of Aerobic Phosphorylation*

All samples contained 1.0 cc of dialyzed phosphate extract, 0.005 M  $MgCl_2$ , 0.001 M adenosine triphosphate (0.1 mg. of acid-labile P), 0.025 M NaF, and either 0.037 M glucose or 0.042 M creatine. In addition, the aerobic samples contained 0.036 M pyruvate, and 0.001 M succinate; the anaerobic samples contained 0.0007 M cozymase, 0.036 M pyruvate, 0.009 M hexose diphosphate (0.8 to 1.0 mg. of P), and 0.001 M  $NaHCO_3$ . Temperature 37°. Gas phase, aerobic 100 per cent oxygen; anaerobic, 95 per cent nitrogen and 5 per cent carbon dioxide

Experiment No	Phosphate acceptor	Aerobic reaction				Anaerobic reaction						P esterified per 1 equivalent oxygen atom reduced (P:O)		Esterification ratio, aerobic/anaerobic $\frac{(a)}{(b)}$
		Incubation time	R Q	Equivalent oxygen reduced*	Phosphate esterified	Incubation time	Equivalent oxygen reduced				Phosphate esterified	Aerobic (a)	Anaerobic (b)	
							Pyruvate removed	Lactate formed	Phosphoglycerate formed	Mean				
		min		micro-atoms	micro-atoms P	min	micro-moles	micro-moles	micro-moles	micro-moles	micro-atoms P	atoms	atoms	
1	Glucose	25	1.31	19.6	48.5	20	26.0	22.5		24.2	15.2	2.5	0.63	4.0
2	"	25	1.41	15.4	37.4	20	18.2	17.4		17.8	12.6	2.4	0.71	3.4
3	"	26	1.11	37.8	55.5	20	33.3			33.3	19.7	1.5	0.59	2.5
4	"	35	1.16	13.2	24.0	30	27.0			27.0	17.1	1.8	0.63	2.9
5	"	31	1.17	16.4	37.4	26	22.3	24.4		23.3	15.0	2.3	0.64	3.6
6	"	20	1.11	25.4	24.5	15	17.4			17.4	7.1	0.96	0.41	2.3
7	Creatine	26	1.17	18.8	28.7	20	20.0	21.5	19.0	20.2	10.3	1.5	0.51	2.9
8	"	35	1.21	11.6	26.8	10		12.9	12.0	12.5	11.3	2.3	0.94	2.4
9	"	18	1.10	21.4	43.5	12	18.2	18.2	18.4	18.3	11.0	2.0	0.60	3.3
10	"	20	1.14	29.2	48.0	10	14.6	13.5	11.6	13.2	7.4	1.6	0.56	2.9
Averages . . .			1.19									1.9	0.62	3.1

\* Oxygen uptake.

cooled to 0° prior to the addition of the ice-cold extract and were kept in ice until they were attached to the manometers. The reaction mixtures in the Warburg vessels were equilibrated with either oxygen or nitrogen with 5 per cent carbon dioxide for 2 minutes. At the end of incubation the vessels were rapidly removed and placed in ice; aliquots were then pipetted

into trichloroacetic acid. Zero time values were secured by deproteinizing a sample immediately after addition of the extract. The oxygen consumption of the aerobic samples was corrected to include the uptake during temperature equilibration by assuming that it proceeded at the same rate in this time as during the first 10 minutes of measuring the gas exchange at 37°. In this way some oxygen consumption occurring at a much lower temperature in the short interval that elapsed between addition of the extract to the vessels and the moment when they were placed in the thermostat, as well as during the period between their removal from the manometers and deproteinization of the reaction mixtures, was amply allowed for. The incubation times for the aerobic experiments given in the third column of Table VI include the period of temperature equilibration. Shorter incubation times were chosen in the anaerobic experiments, especially in the ones with creatine, because of the great rapidity with which the dismutation came to an equilibrium in this case.

Respiratory quotients were determined by the method of Warburg and Yabusoe (31). The average value of 1.2 indicates the complete oxidation of pyruvic acid to carbon dioxide and water. The absence of secondary reactions in the aerobic experiments, such as pyruvate dismutation (32), that might contribute to the phosphorylation and thus interfere with the determination of the true P:O values of pyruvate oxidation, is also indicated by the fact that no lactic acid was formed in any of the experiments with creatine, and only traces were formed (*i.e.* 2.3, 2.4, 0.0, and 1.1 micromoles in four experiments in which lactic acid was determined) when glucose was used as phosphate acceptor. The small formation of lactic acid in the latter case was undoubtedly due to the fact that although cozymase was not added there was enough in the extracts so that there occurred some dismutation between the pyruvate (present in excess) and the triose phosphate in equilibrium with the large amounts of fructose diphosphate formed by aerobic phosphorylation of the sugar. It has been previously observed with brain dispersions (3) and heart extracts (33) that the bulk of the phosphorylated glucose remains as the equilibrium mixture fructose diphosphate  $\rightleftharpoons$  2 triose phosphate.

Each of the experiments of Table VI was carried out on triplicate samples, the average values being given in the table. In general variations from the mean did not exceed  $\pm 5$  per cent. A few values that surpassed such variation were discarded and only the average of the two remaining samples was taken.

The direct P:O ratios (Table VI) of individual experiments showed large variations, owing no doubt to the greater or lesser activity of the adenosine-triphosphatase in different extracts. The quotient aerobic P:O to anaerobic P:O had an average value of 3.1.

## DISCUSSION

The results reported in this paper indicate that each of the five primary dehydrogenation steps involved in the oxidation of 1 molecule of pyruvic acid to carbon dioxide and water must generate three phosphate bonds, a total of fifteen phosphate bonds per molecule of pyruvic acid oxidized. On the basis of our present knowledge we can only account for about half of that number. Of the five dehydrogenations of the citric acid cycle (34) carbonyl oxidation in the step  $\alpha$ -ketoglutaric acid + O = succinic acid + CO<sub>2</sub> would account for three phosphate bonds, since it causes as much phosphorylation per atom of oxygen consumed as the complete oxidation of pyruvic acid (35). We are then justified in assuming that the primary oxidation of the carbonyl group of pyruvic acid (whatever its mechanism) may account for another three, but the desaturation step, succinic acid + O = fumaric acid + H<sub>2</sub>O, would seem to account for no more than one phosphate bond (2, 3).<sup>9</sup> As regards the remaining two dehydrogenations, which involve a secondary alcoholic group, the step malic acid + O = oxalacetic acid + H<sub>2</sub>O has been reported to generate enol-phosphate bonds (6) but no quantitative data are available, and nothing is known regarding the step isocitric acid + O =  $\alpha$ -ketoglutaric acid + H<sub>2</sub>O + CO<sub>2</sub>.

No fewer difficulties are encountered if one tries to visualize the generation of three phosphate bonds by carbonyl oxidation in terms of known mechanisms. As recently pointed out by Lipmann (36), there is ample release of energy in the passage of 2 hydrogen atoms from the metabolite at the bottom of the oxidation-reduction scale to oxygen at the top to cover the formation of three energy-rich phosphate bonds, which corresponds to a conversion efficiency of 60 per cent, but it seems unlikely that all the three can be generated within the span of potential between the carbonyl compound and the next hydrogen acceptor, probably alloxazine. Such a span of about 0.52 volt, corresponding to a  $-\Delta F'$  of 25,000 calories, might account at the most for the generation of two phosphate bonds (20,000 to 22,000 calories). This result could conceivably be achieved by the formation of a carboxyl-pyrophosphate bond, a view that may have some support in the finding of a coupling of pyruvate oxidation with synthesis of inorganic pyrophosphate in liver extracts.<sup>10</sup>

At least one of the three phosphate bonds would have to be generated catalytically during the passage of the hydrogens from the potential level of alloxazine protein to that of oxygen, but there is as yet no experimental proof for this assumption. The view that such a function might be performed by the succinate  $\rightleftharpoons$  fumarate system has been previously advanced (2, 3), but the participation of this system in biological oxidations as

<sup>9</sup> Ochoa, S., unpublished.

<sup>10</sup> Cori, G. T., Ochoa, S., and Cori, C. F., unpublished.

a catalytic carrier of hydrogen is now very doubtful (34, 35). Aerobic oxidation of large amounts of dihydrocozymase in heart extracts (involving dihydropyridine, alloxazine, the cytochrome system, and oxygen), in the presence of adenosine triphosphate, inorganic phosphate, glucose, and fluoride, gave a large oxygen uptake but was not accompanied by phosphorylation.<sup>9</sup> Perhaps the generation of three phosphate bonds and their interaction with the adenylic acid system takes place on the same enzyme protein or protein complex when the hydrogens pass through the span of potential between metabolite and cytochrome.

#### SUMMARY

1. Oxidation of pyruvic acid in heart extracts is coupled with phosphorylation of adenylic acid. The phosphorylation product was isolated and identified as adenosine diphosphate.

2. Heart extracts contain a very active adenosinetriphosphatase. The activity of this enzyme was inhibited about 50 per cent by sodium fluoride.

3. Even in the presence of fluoride, the adenosinetriphosphatase in heart extracts interfered with the phosphorylation of glucose by adenosine triphosphate (hexokinase reaction), and with the transfer of phosphate from phosphocreatine to glucose in which the adenylic acid system acts as a catalytic carrier of phosphate between the two substrates. Loss through dephosphorylation varied from 25 to 60 per cent of the phosphate mobilized.

4. Aerobic phosphorylation of phosphate acceptors is the result of (1) phosphorylation of adenylic acid to adenosine polyphosphate, coupled with oxidation, and (2) transfer of phosphate from adenosine polyphosphate to the acceptor. Adenosinetriphosphatase must interfere with (2) as it does with transphosphorylations; hence the P:O ratio of 2 previously obtained for pyruvate oxidation, *i.e.* 2 atoms of phosphorus esterified for every atom of oxygen consumed, must be too low.

5. A comparison of the phosphorylation caused by pyruvic acid oxidation with that produced by the dismutation between phosphoglyceraldehyde and pyruvic acid in heart extracts has given a P:O ratio of 3 for the former reaction. This ratio corresponds to an efficiency of conversion of oxidation energy to phosphate bond energy of nearly 60 per cent. This finding is discussed in relation to present knowledge on pyruvate oxidation and on the mechanisms of generation of phosphate bond energy.

I am very indebted to Dr. E. Bueding for the pyruvate estimations, and to Mrs. Theodora Goldstein for technical assistance.

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# EVIDENCE FOR THE TOTALLY ASYMMETRIC SYNTHESIS OF AMINO ACIDS IN VIVO

BY DAVID SHEMIN AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

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In 1933 Krebs (1) found that animal tissues contain an enzyme, *d*-amino acid oxidase, which catalyzes the oxidative deamination of *d*-amino acids. The function of this highly active enzyme is problematical, for the amino acids of the dietary proteins are, as far as is known, exclusively of the *l* series. The occasional presence of small amounts of *d*-amino acids in protein hydrolysates is readily ascribable to partial racemization during hydrolysis.

Although the amino acids of normal mammalian proteins appear to possess exclusively the *l* configuration, the synthetic processes by which they are produced in the living animal may not necessarily be entirely asymmetric. Should they lead to the formation of racemic amino acids, the *d* components of these might undergo complete deamination under the influence of the *d*-amino acid oxidase.

When a *d*-amino acid is fed to rats, its fate depends on the relative rates of deamination and of excretion. *d*-Tyrosine is a representative of those amino acids which are rapidly deaminized (2); *d*-glutamic acid, which is attacked but slowly if at all by *d*-amino acid oxidase (3), is one of those which are excreted without profound decomposition (4). That *d*-glutamic acid is not normally detectable in urine is an indication, though not proof, that it is not formed in appreciable amounts in the organism. More cogent evidence on this point was sought with the aid of isotopic nitrogen.

If *d*-amino acids are formed by a partial symmetrical synthesis in tissues, and if *d*-amino acids are administered to the animal, the exogenous and endogenous *d*-amino acids should mix and be treated as an entity by the organism. If this assumption is correct and if the ingested amino acid is labeled with heavy nitrogen, the result of such merging would be a decrease in its isotope concentration. Analysis of any excreted *d*-amino acid will then clearly indicate whether the amino acid synthesis in the cell is exclusively asymmetric. Conversely, if isotopic ammonia is administered to rats together with normal *dl*-amino acid, the isolation from the urine of a *d*-amino acid containing  $N^{15}$  may be taken as proof of the synthesis *in vivo* of the *d*-amino acid.

In order to enhance the excretion of *d*-amino acids in the urine, we have

attempted to decrease the amount of *d*-amino acid oxidase in our experimental animals by restricting their intake of riboflavin. Warburg and Christian (5) have shown that the coenzyme of *d*-amino acid oxidase is a flavin dinucleotide. Ochoa and Rossiter (6) and Axelrod, Sober, and Elvehjem (7) have demonstrated that animals on a riboflavin-deficient diet have a lowered *d*-amino acid oxidase content in their tissues.

Isotopic *dl*-tyrosine was fed to vitamin B<sub>2</sub>-deficient rats. The *d*-tyrosine isolated from the urine had the same N<sup>15</sup> concentration as that fed. Similar results were obtained after *dl*-glutamic acid was fed to similarly deficient rats; no dilution of the *d*-glutamic acid was found. This is in agreement with other results of this laboratory, in which, after *d*-glutamic acid had been fed to rats on a complete diet, the *d*-glutamic acid excreted had the same N<sup>15</sup> concentration as that ingested (4).

A more sensitive experiment was carried out in which heavy ammonia, together with *dl*-tyrosine, was administered to vitamin B<sub>2</sub>-deficient rats. If *d*-tyrosine were formed from ammonia as the source of nitrogen, the excreted tyrosine should contain N<sup>15</sup>. None was found.

The results of all of these experiments contraindicate the occurrence of symmetrical synthesis of amino acids by the vitamin B<sub>2</sub>-deficient rat. It seems reasonable to apply this conclusion also to the normal animal.

#### EXPERIMENTAL

*Experiment 1*—A group of rats (average weight 42 gm.) was kept on the following diet: glucose 74 per cent, casein 19 per cent, salt mixture (8) 4 per cent, wheat germ oil 2 per cent, cod liver oil 1 per cent, and 0.2 mg. of vitamin B<sub>1</sub> per 100 gm. of diet. After 8 weeks all the rats showed acute symptoms of riboflavin deficiency. Five rats then received as an addition to their diet 100 mg. of *dl*-glutamic acid hydrochloride per day for 10 days. During this period two rats died. The collected urines were filtered, made 7 per cent with respect to trichloroacetic acid, and filtered. The filtrate was concentrated, an equal volume of concentrated hydrochloric acid was added, and the mixture refluxed for 5 hours. The solution was then taken to dryness *in vacuo*. The residue was dissolved in a small volume of water, and glutamic acid hydrochloride was isolated from the alcohol-insoluble barium salt. The 493 mg. of glutamic acid hydrochloride so obtained were recrystallized twice from 20 per cent hydrochloric acid; yield 324 mg.; N 7.80 per cent;  $[\alpha]_D = -25.1^\circ$ ; N<sup>15</sup> = 0.870 atom per cent excess. The glutamic acid fed contained 0.865 atom per cent excess N<sup>15</sup>.

*Experiment 2*—A group of rats (average weight 45 gm.) was kept on the same diet as in Experiment 1. After 6 weeks all the rats showed acute symptoms of vitamin B<sub>2</sub> deficiency. Six rats then received as an addition to their diet 250 mg. of *dl*-tyrosine, containing 1.00 atom per cent excess

$N^{15}$ , for 3 days. Urine was collected for 9 days after the feeding of *dl*-tyrosine. The urine was acidified to Congo red with hydrochloric acid, filtered, and the filtrate concentrated. Trichloroacetic acid was added sufficient to raise the concentration to 10 per cent, and the mixture was filtered. The filtrate was extracted twice with ether. An equal volume of 20 per cent hydrochloric acid was added to the aqueous phase and the mixture boiled for 3 hours. The solution was taken to dryness *in vacuo*, and the residue dissolved in a small quantity of water and brought to pH 5.6 with 40 per cent sodium hydroxide. After several days at 0° tyrosine crystallized out. After several recrystallizations there were obtained 42 mg. of *d*-tyrosine.  $N$ , calculated, 7.7 per cent; found 7.6 per cent;  $[\alpha]_D^{25} = +10.4^\circ$  (calculated  $+10.6^\circ$  (9));  $[\alpha]_D^{20} = +9.3^\circ$  (calculated  $+9.3^\circ$ );  $N^{15} = 1.03$ , 0.99 atom per cent excess (duplicate determinations); average 1.01.

*Experiment 3*—A group of rats (average weight 38 gm.) was kept on the same diet as in Experiment 1, with the addition of 0.4 mg. of vitamin  $B_6$  and 1 mg. of pantothenic acid per 100 gm. of diet. After 45 days the rats showed acute symptoms of vitamin  $B_2$  deficiency. Six rats then received for 5 days as an addition to their diet 250 mg. of non-isotopic *dl*-tyrosine and ammonium citrate containing 30 mg. of nitrogen with 11.9 atom per cent  $N^{15}$  excess. Urine was collected during this 5 day period and for 2 days thereafter. 107 mg. of tyrosine were isolated from the urine as in Experiment 2.  $[\alpha]_D^{24} = +8.4^\circ$  (calculated  $+10.6^\circ$ );  $N^{15} = 0.018$  atom per cent excess. The optical rotation showed that this tyrosine was not pure. If the impurity were *l*-tyrosine, the sample would contain 10 per cent of this isomer. Pure *d*-tyrosine was obtained by crystallization of the copper salt according to a method developed by Foster.<sup>1</sup> 53 mg. of tyrosine were obtained after this treatment.  $N = 7.7$  per cent (calculated 7.7 per cent);  $[\alpha]_D^{21} = +11.6^\circ$  (calculated  $+11.5^\circ$ );  $N^{15} = 0.004$  atom per cent excess.

#### DISCUSSION

The results obtained in this laboratory when isotopic amino acids were fed to rats show that amino acids are being formed and destroyed at a rapid rate (10). The equality of isotope concentration of the *d*-glutamic acid isolated from the urine with that fed may be taken as proof that little if any *d*-glutamic acid is synthesized by the rat. The amination of ketoglutaric acid to glutamic acid is completely asymmetric. It is difficult to set a lower limit to the amount of *d*-glutamic acid whose synthesis would be consistent with the experimental results. If we assumed that the dietary *d*-glutamic acid completely merges with that formed within the body, then a synthesis of 1 mg. of the *d* isomer per day would have been detectable. These rats averaged 45 gm. in weight and must have contained

<sup>1</sup> Personal communication from G. L. Foster.

about 800 mg. of *l*-glutamic acid. While we have no exact data on the rate at which the glutamic acid in rats is being synthesized and degraded, we cannot be far off in estimating that about 400 mg. of glutamic acid are formed and destroyed per day in these rats. The synthetic reaction must be at least 99 per cent asymmetric.

Similar considerations apply to the results of the experiment in which *dl*-tyrosine was fed. The results of the experiment in which heavy ammonia was fed are the same as those in which labeled amino acids were employed. In the experiment with ammonia containing 11.9 atom per cent  $N^{15}$  excess, and since the  $N^{15}$  concentration of the isolated tyrosine can be determined to  $\pm 0.003$  per cent, even a very slight synthesis of *d*-tyrosine should have been detected. In none of these three experiments is there any indication of a synthesis of *d*-amino acids *in vivo*. Since there seems to be no partial symmetrical synthesis of amino acids from keto acids, the function of the enzyme *d*-amino acid oxidase is still obscure.

It may be pointed out that the technique employed in this investigation is a special application of a general method for the disclosure of intermediates of cellular metabolism. If a substance, labeled with an isotope, is administered to an animal and can subsequently be isolated either from the tissues or excreta, then the isotope concentration of the recovered compound will indicate whether or not it has been diluted by the same non-isotopic substance formed from the dietary constituents. This method has already been employed by Bernhard to demonstrate that the long chain dicarboxylic acids are not normal intermediates in fat metabolism (11).

#### SUMMARY

No synthesis of either *d*-glutamic acid or *d*-tyrosine can be demonstrated in the vitamin B<sub>2</sub>-deficient rat.

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# THE MICROBIOLOGICAL ANALYSIS OF SEVEN AMINO ACIDS WITH *LACTOBACILLUS CASEI*\*

BY S. SHANKMAN, MAX S. DUNN, AND LOUIS B. RUBIN

(From the Wm. T. Thompson Company and the Chemical Laboratory, University of California, Los Angeles)

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The amino acid requirements of *Lactobacillus arabinosus* 17-5 and the analysis with this microorganism of the amino acids cystine, glutamic acid, isoleucine, leucine, methionine, threonine, tryptophane, and valine were described in earlier papers (1, 2) from the authors' laboratories. The analysis with *Lactobacillus casei* of the amino acids arginine, glutamic acid, leucine, phenylalanine, tryptophane, tyrosine, and valine is described in the present paper. A total of eleven amino acids, eight of which are included in Rose's list of essential amino acids, may now be analyzed with satisfactory accuracy by the combined procedures.

The present study is concerned with the quantitative amino acid requirements of *Lactobacillus casei* and methods for the assay of amino acids with this microorganism. Hutchings and Peterson (3) have summarized the literature on the nutritional requirements of *Lactobacillus casei* and have shown that maximum growth results when the microorganism is cultured on a basal medium containing either hydrolyzed casein or a mixture of amino acids.

## EXPERIMENTAL

Stab cultures of *Lactobacillus casei* were carried on yeast extract-dextrose-agar (Difco) and were subcultured monthly. After transfer, the cultures were incubated for 24 to 48 hours at 37° and then maintained in the refrigerator. The inoculum used in the assays was prepared by transferring the stab culture to the riboflavin assay medium (4) in a sterile centrifuge tube and incubating the mixture for 24 hours at 37°. The basal medium of Hutchings and Peterson (3), modified to contain 1 per cent rather than 2 per cent glucose, was used for all of the assays. The biotin employed was a 50 per cent ethanol solution of the S. M. A. Corporation's concentrate No. 5000. Folic acid (the eluate factor) was prepared from The Wilson Laboratories' liver powder 1:20 by the procedure of Hutchings, Bohonos, and Peterson (5). The purification process was carried only through the first norit adsorption and elution, which were sufficient to remove all traces of amino acids.

\* The authors are indebted to Merrill Camien and William Drell for technical assistance.

The accepted experimental techniques described by Snell and coworkers (4, 6) and the experimental procedure given by Hutchings and Peterson (3) were followed throughout. The basal media and the amino acid solutions were adjusted to pH 6.8 by the addition of N sodium hydroxide. The solutions for the analysis of each amino acid were prepared by adding to each of a series of test-tubes 5 ml. of a medium containing all of the substances in the basal medium except the "test" amino acid, 1 to 5 ml. of a standard solution of the "test" amino acid, and sufficient distilled water to make the final volume of the solution 10 ml.

TABLE I

*Effect of Amino Acids on Production of Lactic Acid by Lactobacillus casei*

The maximum acid production per tube varied from 9.0 to 10.5 ml. of 0.1 N acid.

Amino acid	Blank (0.1 N acid formed when each amino acid is omitted)	Concentration of amino acid per test-tube	
		One-half maximum growth	Maximum growth
	ml	$\gamma$	$\gamma$
l(+)-Arginine (free base)* . . . . .	0.60	25	100
Asparagine monohydrate† (natural).	0.8-3.0	Not reproducible	400
l(-)-Cystine* . . . . .	1.0-2.0	" "	2000
l(+)-Glutamic acid* . . . . .	0.4	75	300
l(-)-Leucine* . . . . .	1.1	30	120
dl-Phenylalanine‡ . . . . .	1.2	30	120
dl-Serine* . . . . .	0.8	300	1000
l(-)-Tryptophane* . . . . .	0.7	3	12
l(-)-Tyrosine* . . . . .	1.0	15	60
dl-Valine‡ . . . . .	0.6	40	160

\* Amino Acid Manufactures C.P. or A.P. product.

† Difco.

‡ Merck and Company.

Each assay was run in duplicate at ten levels of the amino acid considered to be most appropriate from an inspection of curves constructed from the data derived from preliminary experiments. For example, the levels of l(-)-leucine employed ranged from 0 to 200  $\gamma$ .

The tubes were plugged with cotton, sterilized by autoclaving for 15 minutes at 15 pounds pressure, and cooled to room temperature. Each tube was inoculated aseptically with 1 drop (0.03 ml.) of a saline suspension of the bacterium and incubated for 72 hours at 37°. The saline suspension of the bacterium was prepared by centrifuging a 24 hour culture of inoculum in a 15 ml. Pyrex centrifuge tube, suspending the cells in sterile 0.9 per cent saline solution, and centrifuging the suspension. The cells were finally suspended in 10 ml. of saline solution. The lactic acid

produced was determined by titration with standard alkali with bromothymol blue indicator. The titrations were reproducible to 0.1 ml. of 0.1 N sodium hydroxide.

The experimental data are summarized in Tables I and II.

TABLE II  
*Experimental Data from Analysis of Eight Amino Acids with Lactobacillus casei*

Amino acid*	Limiting amounts	Amounts present	Amounts found	Deviation
	mg.	mg.	mg	per cent
l(+)-Arginine (free base)	20- 80	55.0	55.2	+0.4
	20- 80	75.1	77.6	+2.5
Asparagine·H <sub>2</sub> O (natural)	25-100	33.2	No data	No data
l(-)-Cystine . . . .	30-120	97.5	" "	" "
l(+)-Glutamic acid	50-200	153.4	152	+0.9
l(-)-Leucine	20- 80	70.8	64	+9.7
dl-Phenylalanine.	20- 80	31.1	29.4	+5.5
dl-Serine. . . . .	200-800	251.2	No data	No data
l(-)-Tryptophane . . . .	20- 80	65.2	63.5	+2.6
l(-)-Tyrosine . . . .	8- 32	26.2	26.2	0
dl-Valine . . . . .	40-160	49.1	48.5	+1.2

\* Amino Acid Manufactures C.P. or A.P. product.

#### DISCUSSION

Although it was observed that little or no growth occurred in the absence of asparagine, cystine, or serine, these amino acids could not be estimated satisfactorily. The curves obtained in several experiments with these amino acids were irregular and varied in slope and position. It is also noteworthy that relatively high concentrations of serine and cystine are required for maximal lactic acid production. In all other cases the standard curves were regular and reproducible.

It may be noted that the amounts of amino acids found differed from those present by percentages ranging from 0 to +9.7 and averaging +3. This average deviation is approximately the same as that found previously by the present authors (2) in the analysis of eight amino acids with *Lactobacillus arabinosus* 17-5. The amino acids leucine, glutamic acid, tryptophane, and valine were analyzed satisfactorily with both microorganisms but a satisfactory analysis of cystine could be obtained only with *Lactobacillus arabinosus* 17-5. In the case of l(-)-leucine, the amount of the amino acid found differed from that present by -0.2 per cent with *Lactobacillus arabinosus* 17-5, and +9.7 per cent with *Lactobacillus casei*. No significance is attached to these results, since it is not improbable that the higher value may be within the limits of probable error of microbiologi-



cal assay. The limiting precision of such methods when applied to amino acids is to be investigated.

#### SUMMARY

Methods have been described for the microbiological analysis with *Lactobacillus casei* of the amino acids arginine, *glutamic acid*, *leucine*, phenylalanine, *tryptophane*, tyrosine, and *valine*. Procedures for the microbiological analysis with *Lactobacillus arabinosus* 17-5 of the four italicized amino acids and four additional ones were previously described by the present authors (2). A total of eleven different amino acids may now be analyzed satisfactorily by the combined methods.

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# DETERMINATION OF CARBONIC ANHYDRASE IN HUMAN AUTOPSY TISSUE

BY WINIFRED ASHBY AND DOROTHEA V. CHAN

(From the Blackburn Laboratory, Saint Elizabeths Hospital, Washington)

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In 1932 Meldrum and Roughton (1) isolated from mammalian red blood cells a highly active enzyme which they called carbonic anhydrase. This enzyme catalyzes the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  and is responsible for the accelerated excretion of  $\text{CO}_2$  from the blood. In any assay of the enzyme in tissues the presence of blood has presented difficulties. In this report a new adaptation of a method for measuring the blood content of tissues is described.

Two general methods for determining carbonic anhydrase are in use, (1) that of Meldrum and Roughton (1) in which the forward reaction (above) is measured, and (2) that of Philpots and Philpots as modified by Keilin and Mann (2) and that of Brinkman (3) in both of which the rate of the reverse reaction is measured. The unit of carbonic anhydrase is defined as that concentration which will halve the time of an uncatalyzed reaction. The standard conditions have not been established.

The magnitude of the unit is affected by temperature, since Hodgson (4) has found that a rise of  $10^\circ$  increases the uncatalyzed reaction 3.6 times and the catalyzed reaction 2.1 times. At  $37.5^\circ$  the differences between the two reactions, therefore, become greatly lessened, rendering the determination of the enzyme activity less sensitive.  $15^\circ$  or the temperature resulting from melting ice has usually been used. Other variables, which affect the unit value and cause discrepancies in the results, are the rate of shaking and probably also the size of the boats used in the Meldrum and Roughton method, as well as the degree of agitation and rate of flow of the carbon dioxide in the Keilin and Mann modification of the method of Philpots and Philpots. Dilution, too, markedly affects the size of the unit by the latter method.

In an effort to meet the difficulty introduced by the presence of blood in the tissues, Brinkman (3), Ferguson, Lewis, and Smith (5), and van Goor (6) have worked with invertebrates whose blood contained little or no carbonic anhydrase. Others (7) have perfused the organ in order to remove as much blood as possible. In other instances the tissues have been extracted (sometimes after perfusion), the extract filtered, and the hemoglobin matched colorimetrically against the animal's blood. Allowance was then made for the enzyme equivalent of the blood in the tissues.

## EXPERIMENTAL

*Preparation of Enzyme Emulsion*—Tissues which were obtained as soon as practicable after death were sealed in parafilm and kept in the freezing chamber of a refrigerator (gross bacterial contamination was prevented). The frozen tissues of the central nervous system were ground between glass plates, or the other tissues when necessary between stones, and a smooth emulsion was made by slow addition with stirring of 9 parts of water followed by dilution to 1:20 with physiological saline. Any further dilutions were made with 0.425 per cent saline. The whole emulsion was used, since in seven experiments with brain emulsions it was found that 30 per cent of the total carbonic anhydrase activity was lost from the supernatant fluid by sedimentation of the particulate matter.

*Determination of Blood in Tissue Emulsion*—The amount of blood in the tissue was determined with the benzidine reaction by comparing known dilutions of packed red cells from the body from which the tissues in question were taken with dilutions of the tissue emulsion. The red cells were hemolyzed with water, followed by further dilution with an equal amount of physiological saline. To 1 cc. each of tissue emulsion and hemolyzed blood dilution was added 1 cc. of a saturated solution of benzidine,  $C_{12}H_{12}N_2$ , (the dihydrochloride is unsatisfactory) in glacial acetic acid. To each mixture 2 cc. of 3 per cent hydrogen peroxide were added simultaneously. The two dilutions of hemolyzed red cells and tissue emulsion were so adjusted that the developments of color were parallel both with respect to time and degree. A light, but substantial, green was chosen as the end-point.

By using the benzidine technique, a specimen of the cortex of kidney in a dilution of 1:4000 was found to have the same hemoglobin content as a 1:50,000 dilution of packed red corpuscles. This indicated that the tissue consisted of 8 per cent red blood cells or approximately 16 per cent of blood. In brain the balanced dilutions might be 1:200 for brain emulsion equal to 1:40,000 for blood, giving 0.5 per cent blood in the brain.

The much smaller quantity of blood which was found in the brain by the benzidine method than in other tissue was not due to any inhibition of the benzidine reaction by brain material. Known quantities of blood added to ground brain and thoroughly intermixed were recovered immediately after being added and after 4 and 7 days storage.

*Determination of Carbonic Anhydrase*—The technique was essentially that of Philpots and Philpots as modified by Keilin and Mann, in which the increased rate of the reaction of carbon dioxide with water to form carbonic acid is measured under the influence of the enzyme. A steady<sup>1</sup> flow of  $CO_2$

<sup>1</sup> A needle valve, covered with an electric pad to prevent irregularities due to ice particles, was used.

from a cylinder of carbon dioxide was run into 10 cc. of 0.0026 M  $\text{NaHCO}_3$ , containing 0.2 cc. of 0.04 per cent phenol red and the enzyme emulsion, until the mixture was just acidified. Then the standard, 2 cc. of 20.6 per cent M  $\text{NaHCO}_3$  + 30 per cent M  $\text{Na}_2\text{CO}_3$ , was added and the time required for acidification was accurately measured in triplicate with a stop-watch. In the uncatalyzed reaction the flow of  $\text{CO}_2$  was adjusted to acidify the

TABLE I

*Carbonic Anhydrase of Tissue from Adjacent Sections of Central Nervous System*

Case No.	Source of tissue	R b c content of tissue	Carbonic anhydrase found per gm.		
			Total	R.b.c.	Net
		<i>per cent</i>	<i>units</i>	<i>units</i>	<i>units</i>
8052	Frontal pole	0.43	30.2	2.2	28.0
		0.5	29.3	2.6	26.7
8064	" "	1.25	43.8	10.0	33.8
		1.25	40.8	10.0	30.8
	" "	1.11	44.5	8.8	35.7
		0.83	40.0	6.6	33.4
		0.67	24.7	5.3	19.4
7995	Cord	0.71	24.2	5.7	18.5
		0.38	24.4	2.6	21.8
	Temporal pole	0.38	24.1	2.6	21.5
		0.73	26.3	5.0	21.3
	Parietal lobe	0.54	25.9	3.7	22.2
		1.11	22.7	7.6	15.1
	Occipital pole	1.23	21.8	8.2	13.6
		1.25	29.7	8.5	21.2
7991	Cerebellum	0.38	22.1	2.6	19.5
		1.15	39.1	8.0	31.1
		1.1	35.8	7.7	28.1
8077	Occipital pole	1.1	35.8	7.7	28.1
		0.63	35.8	7.7	28.1
	Frontal pole	0.63	39.0	4.0	35.0
		0.63	39.0	4.0	35.0
	Parietal lobe	0.61	45.0	3.9	41.1
		0.63	44.7	4.0	40.7
	Occipital pole	0.81	50.7	5.1	45.6
		0.83	50.0	5.3	44.7
	Corpus callosum	1.11	38.0	7.0	31.0
		1.11	35.0	7.0	28.0

standard in 66 seconds. The amount of enzyme emulsion which reduced this time required for acidification to 33 seconds contained 1 unit of carbonic anhydrase. The amount of emulsion which contained 1 unit was determined and from this the amount per gm. of tissue. The reagents were kept in chopped ice until used and the determinations were made in water held at approximately 3°. The reaction mixture was agitated with mechanical regularity.

By means of this technique with 0.5 cc. of a 1:80 dilution of kidney cortex the standard was acidified in 35.6 seconds, while with 0.6 cc. 32.7 seconds were required. By interpolation, 1 unit of carbonic anhydrase was found in 0.59 cc. of this emulsion or 1 gm. of kidney cortex contained 135 units.

*Reproducibility of Technique*—When the technique was applied to tissues obtained from adjacent convolutions of the human cerebrum and cerebellum, results which differed by an average of 5 per cent were obtained (Table I). The units of carbonic anhydrase per gm. of tissue were obtained by subtracting the units of carbonic anhydrase which were given by the amount of blood found in the tissue from the total carbonic anhydrase of the tissue.

*Effect of Dilution upon Sensitivity*—In addition to the effect of temperature, discussed by Roughton and measured by Hodgson, we noted that with a given amount of standard to be acidified neutralization is more rapid in a greater amount of liquid, owing of course to the fact that with adequate

TABLE II

*Apparent Variation in Unit Value of 0.7 Cc. of Brain Emulsion Run in 5, 10, and 20 Cc. of Diluting Fluid with 2 Cc. of Standard*

Total volume	Time			Units
	Catalyzed	Uncatalyzed		
cc.	sec.	min.	sec.	
7.7	35 4	1	34.0	1.33
12.7	34 0	1	3.9	0.97
22.7	28.6		39.6	0.69

CO<sub>2</sub> more carbonic acid is produced in the presence of more water. In the presence of the enzyme, however, the change in rate with dilution is comparatively slight. As a result the same amount of brain emulsion, 0.7 cc., tested with 2 cc. of the standard in 20 cc. of fluid appeared to contain only half as much enzyme as when tested in 5 cc. of fluid. Results with three different amounts of fluids are given in Table II.

## SUMMARY

We have described a procedure, based on the technique of Philpots and Philpots as modified by Keilin and Mann, appropriate for determining the carbonic anhydrase content of human tissues obtained at autopsy.

As a departure from previous techniques, the benzidine reaction has been used to titrate the blood content of the tissue and an emulsion of the whole tissue ground to particles of cellular dimensions has been used in place of extractions.

The reproducibility of the technique is illustrated by complete determinations on adjacent portions of the central nervous tissue which indicate an average difference of 5 per cent.

An effect of increasing sensitiveness, as the test is conducted in smaller volume, is noted.

The small amount of blood found in the brain by the benzidine method has been verified by the recovery of known amounts of blood added to ground brain.

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# CARBONIC ANHYDRASE IN MAMMALIAN TISSUE

By WINIFRED ASHBY

WITH THE TECHNICAL ASSISTANCE OF DOROTHEA V. CHAN

(From the Blackburn Laboratory, Saint Elizabeths Hospital, Washington)

(Received for publication, July 8, 1943)

The purpose of this work is primarily to study carbonic anhydrase distribution in the central nervous system, the results of which will be given in subsequent papers. The present paper deals with an exploration of the possibilities of the technique previously described (1) by use of tissues, some of which, like those of the central nervous system, do not have specific excretory or secretory functions with reference to  $\text{CO}_2$ , the  $\text{H}^+$  ion, or the  $\text{CO}_3$  anion. The aim has been to establish an orientation towards the function of the carbonic anhydrase found in the central nervous system.

The function of carbonic anhydrase in the blood as an accelerator of the elimination of  $\text{CO}_2$  in the lungs has been advanced by Roughton (2). In the goat fetus the enzyme does not appear until shortly before term (3). Carbonic anhydrase has been found in the kidney of the dog, cat, and rat, where it is considered probable that it functions in the excretion of acid (4). In the gastric mucosa, it is thought to be functionally related to the production of gastric  $\text{HCl}$  (5-7). In the pancreas the production of carbonate in the pancreatic juice is attributed to its activity (8, 9), but intravenous injection of sulfanilamide in amount adequate for inhibition of the enzyme did not inhibit normal secretion of pancreatic juice (10).

In the study of organs which do not have a specific excretory function it has been found in the brain of the rat in small amount (9), in the retina of fishes (11), in the lens and retina of the ox (12), and in the spleen (8). In the muscle of the squid, an animal whose blood contains a negligible amount of carbonic anhydrase, from 30 to 50 units per gm. were reported (13), while none was found in the leg muscle of the rabbit after perfusion to remove the blood (3).

## EXPERIMENTAL

*Carbonic Anhydrase Content of Blood of Various Mammalian Species*—The technique previously described (1) was used. The carbonic anhydrase content of the blood of several species of animals is given in Table I. These are compared with the findings of other authors for the same species. If absolute amounts are considered, no agreement is found between these results. There is, however, a rough proportionate agreement from species to species. Because of this lack of a means to express results quantitatively



on any absolute basis, the carbonic anhydrase content of tissues which follow has been related to the blood carbonic anhydrase of the animals from which the tissues were taken.

TABLE I

*Comparison of Carbonic Anhydrase Activity Found in Blood of Various Species of Animals by Different Investigators*

The results are expressed in units of anhydrase (cf. (1)) per c.mm.

	Hodgson (14); 15°, whole blood	Davenport and Wilhelmi (4); 0°, whole blood	Van Goor (9); 0°, whole blood	Author's findings 3°, r b.c	Meldrum and Roughton (3), 15°; whole blood
Rat			8.2	1.44	1.70
Cat..		3.73		1.28	
Dog ...		3.10		0.84	
Cattle....			5.43	1.4-0.84	1.10
Man.....	1.55		5.00	0.73	0.55
Pig.....			4.62	0.59	
Guinea pig			4.50	0.60	
Calf...				0.44	
Chicken. .				0.22	

TABLE II

*Carbonic Anhydrase of Kidney Cortex and Medulla Compared with That of Blood*

Kidney	Blood content of tissue	Carbonic anhydrase		Ratio, units per gm tissue to units per cc. whole blood
		Total	Tissue	
	<i>per cent</i>	<i>units per gm</i>	<i>units per gm.</i>	
Human, Case 8055, medulla	20	110	64	14:100
“ “ 8055, cortex	17	127	87	19:100
“ “ 8053, medulla	20	85	27	9:100
“ “ 8053, cortex	13	97	58	20:100
“ “ 8051, medulla	20	96	46	18:100
“ “ 8051, cortex	16	107	67	26:100
“ “ 7995, medulla	11	85	46	13:100
“ “ 7995, cortex	7	102	78	23:100
“ “ 7983, mixed	10	102	67	20:100
“ “ 7986, “	14	98	44	13:100
Wild rat, mixed	12	130	80	20:100
Guinea Pig I, mixed	20	42.5	9	3:100
“ “ II, medulla	6.6	7.0	-7	-3:100
“ “ “ cortex	16	47	12	5:100

*Carbonic Anhydrase in Kidney*—Findings for the carbonic anhydrase content of the kidney are given in Table II. The per cent of blood which the organ retained after removal of gross blood is indicated. The total carbonic

anhydrase activity per gm. of tissue and the net value obtained by subtraction of the carbonic anhydrase attributable to the blood content are given. The latter is compared with the carbonic anhydrase content of the homologous blood cells collected in citrate, packed by standard centrifuging, and calculated as whole blood on the basis of a 50 per cent dilution. The data, it will be seen, show carbonic anhydrase activity attributable to the human kidney tissue, both of the cortex and of the medulla, but the enzyme was found in greater amount in the cortex. The ratios between the carbonic anhydrase content of the cortical tissues and of the blood averaged 22:100, while those for the medullary tissues and the blood averaged 13.5:100. The kidney of the rat showed about the same relative carbonic anhydrase content as that of man, while values for the guinea pig were considerably lower, with a negative result for the medulla in the one instance in which this tissue was separated from the cortex.

The findings for man differ from those of Davenport and Wilhelmi for the dog and cat in that I obtained a smaller proportionate amount of carbonic anhydrase, while Davenport and Wilhelmi obtained evidence of amounts equivalent to that of the animal's blood in the case of the cat. Also, in the present work the enzyme was found in the medulla of the kidney of man as well as in the cortex, although in a lesser amount, whereas Davenport and Wilhelmi found none in the kidney medulla of the dog and cat.

*Carbonic Anhydrase in Liver and Muscle*—In Table III are given the data for liver and muscle. In these tissues a wide variation in the amount of carbonic anhydrase attributable to the tissue cells is found. The per cent of blood found in the tissues is only crudely significant, especially so with respect to the liver, as the amount remaining in the specimen has been influenced by the preliminary treatment. The ratios between the carbonic anhydrase of the liver tissue and that of the blood in the case of the rat, cat, and guinea pig are very low. In the case of one guinea pig there is a minus quantity. To what extent the inhibiting effects (15) of the sera of these animals upon the enzyme come into play in producing these results has not been ascertained. In the three human liver tissues a carbonic anhydrase activity was found ranging from 7 to 13 per cent of that of the respective blood.

In the human muscle great irregularity of result is obtained, ranging from 4 units per gm., which is practically zero, to 72 units per gm., which constituted 28 per cent of that found in the blood. In the two instances in which the muscle from the neck (which was usually the sternomastoid) and the muscle from the chest (which was usually part of the pectoral muscle) were obtained from the same individual, the sternomastoid was found to have about half the carbonic anhydrase activity of the pectoral

muscle. Low degrees of activity were found in the muscles of a patient dying of tuberculosis (Case 8052), carcinoma of the stomach (Case 8112), and carcinoma of the rectum (Case 8053). In one instance of neoplastic disease of unknown origin (Case 8102), in which the general physical deterioration was extreme, the carbonic anhydrase activity was practically zero. It was also low in Case 7991, a woman of 82 who died 2 weeks after an apparent recovery from pneumonia. The average in these instances was 14.7 units per gm., while for the remaining cases, with death in three

TABLE III

*Carbonic Anhydrase in Tissue of Liver and Striated Muscle Compared with That of Blood*

	Age	Blood content of tissue	Carbonic anhydrase		Ratio, units per gm. tissue to units per cc. blood
			Total	Tissue	
Liver					
	yrs.	per cent	units per gm.	units per gm	
Human, Case 7983	76	12	61.5	22.5	7:100
“ “ 7995	45	22	100	26	8:100
“ “ 7991	82	24	129	46	13:100
Norway rat		20	93	10	1:100
Cat		12 3	111	28	4:100
Guinea Pig I		54	124	7	1:100
“ “ II		8	16.6	-11.4	
Muscle (human)					
Neck, Case 7991, F.	82	5.3	29.8	12.4	4:100
“ “ 8051, “	69	5	44	31	12:100
Chest, “ 8051.	69	13.5	116	72	28:100
“ “ 8102, M.	66	13	46	4	1:100
Neck, “ 8055, “	61	10	69	46	20:100
Chest, “ 8112, “	56	6.6	53	13	2:100
Neck, “ 8053, “	57	10	46	17	6:100
Chest, “ 8053.	57	10	61	32	11:100
Groin, “ 7886, M.	53	7	67	41	12:100
Neck, “ 8052, “	45	5	26.8	13.8	5:100

instances from pneumonia and in the fourth from aortic insufficiency, the average for the muscle tissue was 47.5 units per gm.

*Carbonic Anhydrase in Adrenal and Embryonic Tissue*—The study of the adrenal gland gave consistent results which indicated no carbonic anhydrase in the tissue of that organ. Carbonic anhydrase determinations were also made on rat embryos with results which indicated no carbonic anhydrase. The results are given in Table IV. The average blood content for the adrenal tissue was somewhat over 5 per cent.

*Carbonic Anhydrase in Brain*—Of the tissues studied in this series, the most consistent in its carbonic anhydrase content was the central nervous system, provided areas of highest content were compared. As will be shown subsequently, the carbonic anhydrase activity of the central nervous

TABLE IV  
*Tissues Giving No Evidence of Carbonic Anhydrase Activity*

	Carbonic anhydrase	
	Total	Tissue
	units per gm.	units per gm.
Human, adrenal gland	25.1	0.0
" " " " " "	9.7	-6.1
" " " " " "	12.2	1.7
" " " " " "	16.8	-2.0
" " " " " "	19.5	0.8
Hog, " " " " " "	2.0	-4.0
Rat, embryos	12.6	-4.1
" " " " " "	4	-4.2

TABLE V  
*Comparison of Maximum Carbonic Anhydrase Content of Central Nervous System with That of Blood*

Species	No. of specimens	Area of maximum enzyme content	Carbonic anhydrase	
			Average units per gm. tissue	Ratio, units per gm tissue to units per cc. blood
Man*	3	Cerebrum	38.3	10.5:100
Cat	2	"	47.5	7.4:100
Hog	2	"	34.1	11.6:100
Sow	2	"	23.6	8.0:100
Dog	1	Cerebellum	47.0	11.2:100
Rat, white	3	"	101.0	13.0:100
" Norway	3	"	60.0	14.0:100
Guinea pig	6	"	37.2	12.4:100
Steer	1	"	35.4	8.8:100
Calf	1	"	25.3	11.4:100
Chicken	5	"	29.5	12.4:100

\* Average of frontal and occipital poles.

system varies with the area from which the tissue is taken and in certain species the cerebrum shows more enzyme activity than the cerebellum, while in others the reverse is true. In Table V is given the activity of the cerebrum or the cerebellum, depending upon which shows the greater enzyme content. This is compared with the activity of the animal's

blood. The blood content of these tissues ranges from 0.8 to 2.5 per cent. The ratio of the carbonic anhydrase activity of the tissues, after that attributable to the blood content has been subtracted, ranges from 7.4 to 14 per cent of that of the animal's blood.

#### DISCUSSION

The tissues studied fall into three types. There are, first, tissues with no carbonic anhydrase activity not accounted for by their blood content: the adrenal and rat embryos. Second, are the tissues in which much variation in the enzymatic activity is found; these are represented by the medulla of the kidney, the liver, and the striated muscle of man. In the last group was found the wide range of 1:100 to 28:100 of tissue activity to blood activity, with a decreased value in this series associated with malignancy and extreme old age. Third, there are areas of the central nervous system, either cerebrum or cerebellum in which, through a range of eight different species, a relatively constant relationship holds between the carbonic anhydrase of the tissue and that of the blood, 7.4:100 to 14.0:100.

Roughton expresses the opinion that with  $\text{CO}_2$  as the last stage of metabolism, abundant carbonic anhydrase in the tissue would cause the formation of the hydroxide from  $\text{CO}_2$  and, because of the relative impermeability of the cell membrane to the  $\text{CO}_3$  ion, prevent its diffusion from the cells. Von Bakker, who found the enzyme in considerable amount in the lens of the eye, postulates that, with a poorly developed vascularity, there would be need for a high concentration of carbonic anhydrase to catalyze the dissociation of carbonic acid formed.

Neither of these opposed theories, however, fits the data here presented, for in the adrenal tissue no carbonic anhydrase was found, while the tissue of the brain exhibited a substantial enzyme activity through the range of all species of animals studied; and both of these tissues may safely be assumed to have an adequate blood supply during life, even if, as my data indicate for the brain, the tissues of this organ become comparatively empty of blood at death. I offer, as an alternative hypothesis, one which attributes to carbonic anhydrase a function associated with the progress of events within the chain of interrelated reactions peculiar to the metabolism of the tissue in question, rather than a function pertaining to the excretion of the end-product  $\text{CO}_2$ .

#### SUMMARY

1. The carbonic anhydrase content of the following tissues has been studied by the technique previously described by which the carbonic anhydrase activity of the tissues can be distinguished from that of the contained blood: kidney divided into medulla and cortex, liver, human striated muscle, adrenal, rat embryo, and brain.

2. In the human kidney the enzyme of the cortex was higher than that of the medulla. The medulla contained variable amounts of the enzyme. In this series of human muscle low activity was associated with malignancy, tuberculosis, and extreme old age, and greater activity after a comparatively rapid death. Variable results both between species and individuals were also found in other tissues.

3. In contrast to the above, no activity was found in the adrenal or in the rat embryo, while an activity approaching 10 per cent of that of the blood was found as a maximum in the central nervous system of eight species of animals studied.

4. The possible significance of carbonic anhydrase in the brain is discussed.

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# UREA IS NOT EQUALLY DISTRIBUTED BETWEEN THE WATER OF THE BLOOD CELLS AND THAT OF THE PLASMA

By J. O. RALLS

WITH THE TECHNICAL ASSISTANCE OF E. B. CROHN

(From the Laboratories of Biological Chemistry, University of Buffalo School of Medicine, Buffalo)

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A graphic analysis of some data on the correlation between blood urea concentrations and urea output in man was made by the author some years ago.<sup>1</sup> That analysis led to the conclusion that a cubical parabola was the type of curve that fitted the data best. The curve had an intercept on the blood urea axis at about 9 mg. per cent. While this was suggestive of a threshold, it was known that, with perhaps one exception (1), the idea of a urea threshold had been rather generally rejected (2-5). To us it seemed, however, that at least some of the urea was not freely excreted and existed, perhaps, as a urea complex, or was loosely bound in the blood.

Such "bound urea," whether in the red blood cells or in the plasma, should cause a lack of freedom in the diffusion of urea and an unequal distribution of that substance between the corpuscles and the plasma. This would be contrary to the commonly accepted dicta that urea is freely diffusible and that "urea is about equally divided between the water of the corpuscles and that of the plasma" (6). When the old works (7-16) which were often quoted in support of the equal distribution concept were examined, it was found that, with but one exception (15), no consideration had been given to the difference in the water content of the cells and of the plasma. In that one exception, Ege's view that there is about 80 per cent as much water in the cells as there is in the plasma (17) was used as the basis for the comparison of the urea concentrations of those two blood phases. Using all the old data mentioned above, we recalculated the urea concentrations in the water of the cells and in that of the plasma, making use of the average figures 71 and 93.4 volumes per cent of water in cells and plasma, respectively, and (when cell volumes had not been given) 46 volumes per cent of cells in whole blood. From these new concentrations, cell urea to plasma urea ratios were calculated. Similar ratios were calculated from the data of Boyd (18) who had actually directly determined cell urea and plasma urea but had not been interested in the water concentrations. All the ratios were then plotted in a frequency

<sup>1</sup> Griffith, F. R., Ralls, J. O., and Pucher, G. W., unpublished.



barograph (Fig. 1). It was immediately evident that there was little or no basis for the contention that urea is equally divided between the cell water and the plasma water. Recently, at least one worker (19) has maintained that the ratio of the urea in the *free* water of the cells to that in the *free* water of the plasma varies between 1.06 and 1.96. Thus it seemed to us that the question of the distribution of urea between cells and plasma was by no means settled and we felt justified in continuing our studies along that line. This paper is a report of our findings.

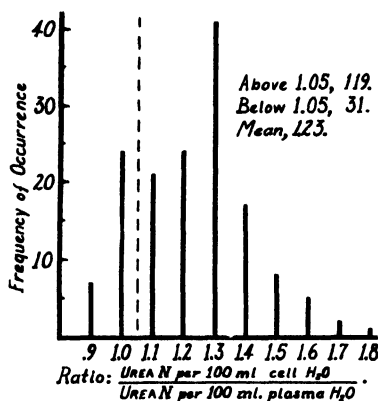


FIG. 1. A barograph showing the frequency of occurrence of various ratios between the concentration of urea nitrogen in the cells and its concentration in the plasma (allowance for the water content of the two phases being made) as calculated from data in the literature.

#### EXPERIMENTAL

77 samples of blood were contributed by 66 individuals (forty-nine males and seventeen females) within the age range of 20 to 40 years. 66 samples were taken under postabsorptive conditions and eleven were taken under absorptive conditions. The blood was taken by venipuncture without stasis. Heparin was used as the anticoagulant in order to avoid changes in cell volume and any possible shift of urea from the cells to the plasma (18). Plasma was obtained by immediately centrifuging a portion of each blood sample.

Urea nitrogen and total water were determined on the whole blood and the plasma, while cell volume was obtained by hematocrit.

#### Methods and Errors

*Cell Volume*—The hematocrit tubes were spun at 3000 R.P.M. for 40 minutes. The results on a single sample of blood were 44.00, 43.96, 44.12,

43.87, 43.85, 43.76, 44.10, 43.99, 43.90, and 43.81, averaging  $43.9 \pm 0.15$ , with a maximum spread of 0.3 per cent. In all calculations, the cell volume was rounded to the nearest 0.1 per cent.

*Total Water*—A drying and weighing method was adopted after that of Gurevitch and Karlson (20) had been found to be unsatisfactory. A temperature of  $150^{\circ}$  and a drying time of 17 hours were selected, although Nelson and Hulett (21) recommended  $325^{\circ}$ , the critical temperature of water. The samples were measured by means of an Ostwald-Van Slyke pipette (delivering 1.007 ml.) and the temperature of the blood or plasma was noted. After the material was dried, the weight loss was divided by the density of water at the temperature noted. The quotient was corrected by the factor 99.3 ( $1/1.007 \times 100$ ) to give the per cent water in the sample. The results on a single sample of whole blood were 83.3, 83.6, 83.8, 83.9, 83.5, and 83.7 per cent, with a maximum deviation of 0.6 per cent. The results on a single sample of plasma averaged 93.4 per cent, with a maximum spread of 0.3 per cent.

*Urea Nitrogen*—This quantity was determined by the manometric method of Van Slyke (22, 23), but with certain modifications described below.

Because difficulty was experienced in obtaining sufficiently accurate pressure readings at the 0.5 ml. mark, the practice of measuring all pressures at the 2.0 ml. graduation was adopted. This practice eliminated the major part of the error introduced when a small bit of stop-cock grease got into the capillary just below the upper stop-cock. A 0.01 ml. piece of grease at this point introduces an error of 2 per cent when the pressures are read at 0.5 ml., whereas, when they are read at the 2.0 ml. mark, just a 0.5 per cent error is introduced. As an example of the troubles thus avoided, the following pressure readings on the same quantity of trapped air are given, with the usual 2 minute extraction period preceding each reading: at 0.5 ml. and  $28.5^{\circ}$ , 31.64, 31.97, 31.80, 31.98, 31.78, 31.98, 31.65, 32.02, 31.89, 31.95, 31.73, 31.70, 31.80, 32.02, 31.95, 31.78, 31.92, and 31.98, maximum deviation 0.38 cm.; at 2.0 ml. and  $28.5^{\circ}$ , 13.12, 13.13, 13.12, 13.14, 13.12, 13.14, 13.12, 13.14, 13.14, 13.12, 13.12, 13.11, 13.14, 13.14, and 13.13, maximum deviation 0.03 cm. Thus it was evident that, although the urea nitrogen factor per mm. of carbon dioxide pressure at 2.0 ml. is approximately 4 times that at 0.5 ml., a considerable net gain in accuracy was effected.

It was found necessary to run frequent blanks during each day, because these invariably increased as the day progressed. Howell (24) also found that changes in the blank occurred as the age of the urease solution increased.

In practice, the time at which the urease solution was added to the reac-

tion chamber was noted in all blank and experimental determinations. The blanks for experimental determinations were obtained by interpolation between the preceding and succeeding blanks. The urease solutions were prepared from Squibb's Double Strength urease powder.

*Correction Factors*—The reaction chamber was calibrated at the 2.0 ml. mark and was found to have an actual volume of 1.988 ml. This introduced a correction factor of 0.994, which in conjunction with that of 0.993 for the pipette gave an over-all factor of 0.986. All our determinations of urea nitrogen were corrected by means of this last factor.

*Check of Procedure and Technique*—For this purpose, we used a solution containing 15 mg. of urea nitrogen and 0.8437 gm. of sodium bicarbonate (equivalent to 45 volumes per cent of carbon dioxide) per 100 ml. The following results were obtained: 14.85, 14.90, 15.10, 14.99, 15.08, 15.02, 15.02, 14.89, 14.91, 15.10, 15.11, 14.85, 14.93, 15.02, 15.00, 15.00, 15.15, 14.85, and 14.86, with an average of 14.98 mg. of urea nitrogen per 100 ml. The maximum range was  $\pm 1$  per cent of the correct value.

*Effect of Each Error on Ratio of Urea N per 100 Ml. Cell Water to Urea N per 100 Ml. Plasma Water*—An absolute error of  $\pm 0.3$  per cent in the cell volume introduces a negligible error of  $\pm 0.005$  in the calculated ratio, because the same proportionate error is reflected in both the cell urea nitrogen and the cell water calculations.

An absolute error of  $\pm 0.6$  per cent in the water content of whole blood introduces an error of  $\pm 0.02$  in the ratio, while one of  $\pm 0.3$  per cent in the plasma water introduces an error of  $\pm 0.005$  in the ratio of cell urea to plasma urea.

A relative  $\pm 1$  per cent error in the urea nitrogen of whole blood produces an error of  $\pm 0.03$ , whereas a similar error in the plasma urea nitrogen figure means an error of  $\pm 0.025$  in the ratio.

The most adverse combinations of the above errors (high whole blood urea, high plasma water, low plasma urea, and low whole blood water, or *vice versa*) introduce an error of  $\pm 0.08$  in the ratio of the concentration of urea nitrogen per 100 ml. of cell water to the urea nitrogen per 100 ml. of plasma water.

*Influence of Arginase Activity on Urea Nitrogen Determinations*—Although it was felt that Howell (24) had adequately shown that the "arginase error" in urea determinations resided in an enzyme-substrate system in the urease preparation itself and that we had eliminated that through our use of frequent blank determinations, it seemed advisable, in order to preclude any adverse criticism, to show, if possible, that this "error" was inoperative in our determinations. Accordingly, a study was made of the action of the usual 50 per cent glycerol extract of urease (23) upon arginine carbonate in water and upon blood to which arginine carbonate had been

TABLE I

*Evidence That Action of Arginase Was Insignificant in Our Determination of Distribution of Urea between Human Erythrocytes and Plasma*

Sample No	Description	pH of sample*			pH of reaction mixture*			Urea nitrogen, mg. per 100 ml			Ratio†		
		1 hr ‡	24 hrs	48 hrs	1 hr	24 hrs	48 hrs	1 hr	24 hrs	48 hrs	1 hr	24 hrs.	48 hrs.
1	Aqueous§ arginine carbonate				6.90	6.90		0.00	0.00	0.00			
2	" "				6.91	6.90		0.00	0.00	0.00			
3	" "				6.95	6.94	6.95	0.00	0.00	0.00			
4	Whole blood	7.28	7.36		6.95	7.03		14.08	13.70		1.12	1.12	
4	Plasma from Sample 4	7.29	7.35		6.90	6.95		15.24	14.85				
4a	Whole blood, Sample 4, + arginine carbonate	7.27	7.37	7.36	7.01	7.01	7.02	14.08	15.60	16.70			
4a	Plasma from Sample 4a	7.26	7.33	7.35	6.96	6.95	7.00	15.24	17.00	18.20	1.12	1.12	1.12
5	Whole blood	7.30	7.33		6.92	6.93		9.48	9.03				
5a	" " Sample 5, + arginine carbonate	7.28	7.34		6.90	6.91		9.43	11.33				
6	Whole blood	7.30	7.36	7.32	7.01	7.02	7.02	13.76	13.34		1.12	1.12	
6	Plasma from Sample 6	7.27	7.33		6.97	6.98		14.75	14.31				
6a	Whole blood, Sample 6, + arginine carbonate	7.31	7.40	7.35	6.96	7.00	7.02	13.73	15.25	16.36			
6a	Plasma from Sample 6a		7.30			7.01			17.50				1.12

\* The pH determinations were made by means of a quinhydrone electrode with a type K potentiometer and a glass electrode with a Beckman meter. The values obtained for buffers made to have pH values of 4.00, 6.6, and 7.2 were 4.01, 6.57, and 7.23, respectively. In addition to the pH of the reaction mixtures shown in the table, another whole blood with and without added arginine was tested. The results were 7.00 and 6.92, respectively.

† This ratio is that between the cell urea nitrogen per 100 ml. of cell water and the plasma urea nitrogen per 100 ml. of plasma water.

‡ These times represent the maximum interval from the blood letting and addition of arginine carbonate (or the preparation of the arginine carbonate solution) to the time when the indicated determinations were made.

§ The arginine carbonate solution was made to contain 120 mg. per 100 ml. Analyses showed it contained 32.0 mg. of nitrogen per 100 ml., of which approximately 16 mg. were available for conversion to urea nitrogen by arginase. Arginine carbonate was added to blood to give the same concentration as the above solution. Thus, if the arginase was significantly active, it would have been possible to get an extra 16.00 mg. of urea nitrogen in our blood analyses.

added as compared with its action upon a portion of the same blood to which no arginine had been added. These studies were made under conditions exactly analogous to those prevailing in our urea nitrogen determinations as reported herein. The pH of the reaction mixture in the Van Slyke manometric determination of urea nitrogen, as applied to the above arginine carbonate solutions and the blood with and without added arginine, was determined. The results (Table I) indicate that, under the conditions of our regular determinations, no extra urea was derived from an "arginase action" upon arginine in the blood.

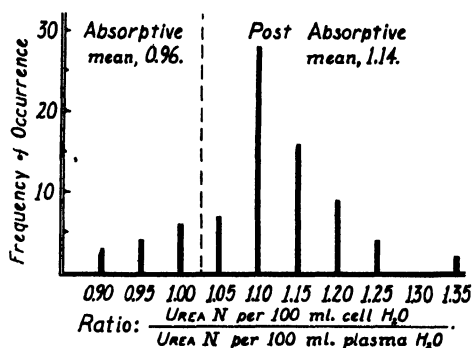


FIG. 2. A barograph showing the frequency of occurrence of various ratios between the concentration of urea nitrogen in the water of red blood cells and its concentration in the plasma water, as determined on the blood of 66 individuals in the post-absorptive state and thirteen in the absorptive state.

### Results

The results of the distribution studies are given in two forms: (1) in a barograph (Fig. 2) depicting the frequency of occurrence of the observed ratios, rounded to the nearest 0.05; and (2) in Table II in which only the averages of the results obtained are given for certain groups of blood: Group A, male and female, 66 postabsorptive samples, Group B, female, seventeen postabsorptive, Group C, male, forty-nine postabsorptive, Group D, male, thirteen postabsorptive, and Group E, eleven samples from the same individuals as in Group D, but in the absorptive state. The ranges, standard deviations, standard deviations of the means, and the probable errors of the means for each group are given.

### DISCUSSION

The average of all the ratios between the concentration of the urea in the total water of the cells and that in the total water of the plasma was  $1.14 \pm \sigma_M = 0.007$ . None of the individual postabsorptive ratios was

less than 1.04, while one was as high as 1.39. If it was assumed that each ratio was 0.08 too high (the result of a combination of the following errors: a relative +1 per cent in the whole blood urea, a relative -1 per cent in the plasma urea, an absolute +0.3 per cent in the plasma water, and an absolute -0.6 per cent in the whole blood water) and each ratio was corrected for this, the average ratio would still be 1.06, or more than 1. Such a combination of errors could not have been operating in the analyses of postabsorptive blood in all 66 samples, nor is it probable that it was operating in all sixteen of those analyses that resulted in ratios as low as 1.04 to 1.08.

The author is well aware of the work of Behre (25), Addis (26), and Anderson and Tompsett (27) who pointed to arginase, either in the blood cells or in the urease preparations, as a source of error in blood urea determinations. Moreover, Edlbacher, Krause, and Merz (28) definitely proved the existence of arginase in blood, while Sumner and Dounce (29), Howell (24), and Hellerman *et al.* (30-32) have certainly shown that an arginolytic enzyme is present in jack bean urease preparations. However, Van Slyke (23), in describing his procedure for blood urea nitrogen, stated that the arginase error was not a factor, while Howell (24), having shown that the error was due to an enzyme-substrate system residing in the urease preparations, suggested means (which we adopted in part) of eliminating it. We are certain that no arginase error was present in our results, because the urease preparation which we used did not demonstrably act upon arginine carbonate during the usual incubation period of a urea nitrogen determination, nor did it liberate any detectable quantity of "extra" urea from blood to which arginine had been added (Table I). This was not surprising in view of the fact that the pH of the reaction mixtures in our urea nitrogen determinations fell within the limits 6.90 to 7.02 and the fact that the pH range of activity of arginase is 7 to 11 (24, 30-34) with an optimum at pH 9.5, or, in the presence of cobaltous ions, at pH 7.5 (31).

According to calculations based upon the various reported velocity constants for the hydrolysis of arginine by arginase at pH 7.5 with added cobaltous ions (30, 31), only 1.2 to 6.5 per cent of any arginine present would be hydrolyzed in the first 3 minutes after the addition of the enzyme. But, under the conditions of our determinations (no added cobalt, a pH of 7.0 instead of 7.5, and 1 to 2 minutes of incubation instead of 3), even less than that fraction of any arginine present would have been hydrolyzed to urea. Even if one assumed that the conditions were ideal for the optimal activity of the arginolytic activity of the jack bean urease preparations and that of the approximately 12.5 mg. of amino acid nitrogen in the red blood cells (35) about 1.25 mg., a high estimate (36, 37), may have been arginine  $\alpha$ -amino nitrogen, there could have been, at most, but

2.5  $\times$  0.06, or 0.15 mg., of "extra" urea nitrogen from that source in our cell urea figures. This amount would have been insufficient to invalidate our calculations of the ratio of cell urea nitrogen to plasma urea nitrogen.

Hence, in view of all the above considerations, it appears certain that urea is not equally divided between the water of the red blood cells and

TABLE II

*Average Analytical Figures, Ratios, and Statistical Data on Blood of Different Groups of Males and Females in Postabsorptive and Absorptive State*

Group	Whole blood			Plasma			Cells			Ratio, $M$ ( $b/a$ )	Statistical data on ratios
	Urea N 100 ml	Per cent wa- ter	Per cent cells	Urea N 100 ml	Per cent wa- ter	Urea N 100 ml water ( $a$ )	Urea N 100 ml	Per cent wa- ter	Urea N 100 ml ( $b$ )		
	mg.			mg		mg	mg		mg		
A	12 63	83 4	45 7	13 46	93 5	14 37	11.65	71 4	16.31	1.14	$R = 1.04-1.39$ $\sigma = \pm 0.065$ $\sigma_M = \pm 0.008$ $E_M = \pm 0.005$
B	13 00	85 0	41 9	13 65	93 7	14 55	11.96	72 7	16.40	1.13	$R = 1.05-1.24$ $\sigma = \pm 0.061$ $\sigma_M = \pm 0.015$ $E_M = \pm 0.01$
C	12 50	82 9	47 0	13 39	93 4	14 31	11 54	70 9	16 28	1.14	$R = 1.04-1.39$ $\sigma = \pm 0.066$ $\sigma_M = \pm 0.009$ $E_M = \pm 0.006$
D	12.80	83 4	45 8	13 75	93.3	14.71	11.73	71.5	16.28	1.12	$R = 1.08-1.16$ $\sigma = \pm 0.024$ $\sigma_M = \pm 0.008$ $E_M = \pm 0.005$
E	13.06	83 6	45 8	14 81	93.4	15.87	10.99	71 8	15.27	0.96	$R = 0.90-1.01$ $\sigma = \pm 0.034$ $\sigma_M = \pm 0.01$ $E_M = \pm 0.007$

Group A, 66 males and females in the postabsorptive state; Group B, seventeen females, postabsorptive; Group C, forty-nine males, postabsorptive; Group D, thirteen males, postabsorptive; and Group E, eleven males from Group D in the absorptive state.  $M$ , mean;  $\sigma$ , standard deviation;  $\sigma_M$ , standard deviation of the mean;  $E_M$ , probable error of the mean.  $(M_D - M_E)/(E_{MD-ME}) = 20$ , the ratio of the difference between means  $D$  and  $E$  to the probable error of that difference.

that of the plasma. In fact, as stated above, we found the average ratio of cell urea per 100 ml. of cell water to plasma urea per 100 ml. of plasma water to be 1.14. It was a matter of further interest to note that there is no significant difference between the distribution of urea in the blood of females and that in the blood of males (Table II).

Although it may be admitted that our data establish that urea is not equally distributed between the total water of the cells and that of the plasma, some might argue that it might be equally divided between the free water of those two blood phases. But, Stolzman (19) has reported evidence to the contrary. He reported ratios varying between 1.06 and 1.96 after having calculated the free water in the cells by means of the relative proportions of chloride in the cells and in the plasma and the assumption that that ion is equally distributed between the free water of those two parts of the blood. However, his calculations are subject to considerable doubt, for others (38-40) have shown that chloride is not so distributed. While we have made no direct determinations of the free water in the bloods analyzed, we have assembled the ratios calculated from

TABLE III

*Ratios of Distribution of Urea between Corpuscles and Plasma, with Total Water and Free Water Content As Bases of Calculation*

Ratio	Basis of calculation
1 14	Our own data on urea and total water
1.13	" " " " " " water, and average protein content of cells and plasma, and degrees of hydration: 0.14 ml per gm. hemoglobin (41); 0.11 ml. per gm. albumin (42); 1.87 ml per gm. fibrin (43); and 1.75 ml. per gm. globulin
1 19	Our own data on urea and total water content, and free water as calculated from degrees of hydration of hemoglobin and albumin given above and degrees of hydration of fibrinogen and globulin (per gm.) assumed the same as for albumin

available data for free water and urea in Table III. It is evident that, at least with the information now at hand, the ratio cannot be made to be the 1:1 that the equal distribution concept demands.

The distribution of urea in blood collected from 1 to 3 hours after breakfast was found to be very different from that in blood from individuals in the postabsorptive state. The average ratio was 0.96 (Group E, Table II). This was, undoubtedly, a reflection of the reported rise in blood urea following a meal (44-47). Folin and Svedberg (15) published data which showed that a preferential increase in the plasma urea occurred after a meal. However, they did not happen to call attention to it. When we took their average figures for urea nitrogen in whole blood and in plasma and, using our average cell volumes per cent and volumes per cent of water in whole blood and in plasma, calculated distribution ratios, we obtained 1.06 and 0.92 for postabsorptive and absorptive samples, respectively. In regard to our own data, it may be said that, although one should not apply



statistical methods to as few as eleven results, such an application here indicates that the chances are better than 1 billion to 1 that the difference between the absorptive and postabsorptive ratios in blood is real. This difference is not unlike, except in magnitude, the difference between the distribution of lactic acid in human blood during glycolysis and that in blood drawn in the basal state (48). During glycolysis, the lactic acid accumulated more rapidly in the plasma than it did in the cells. This observation and ours for urea could, at least in part, be explained as being due to a slow diffusion through the cell membrane. In fact, we found that, when plasma was replaced by mammalian Ringer's solution (containing no urea), the urea nitrogen in the external fluid did not reach a maximum until about 1 hour afterwards. The rate of diffusion of urea into and out of red blood cells is to be more extensively investigated.

The exact cause of the demonstrated accumulation of urea within the red blood cells (in the postabsorptive state) is not known. The polymerization and tautomerization of urea, the rapid removal of urea from plasma at the kidney, the metabolic production of urea within the erythrocytes, and the intracellular fixation of urea by adsorption on or its chemical union with some cell constituent were all considered. The polymerization and tautomerization were discarded as untenable after some consideration. The possibility that a rapid elimination of urea from the plasma and a slow diffusion from the cells might account for the unequal distribution observed was discounted by the observation that, under conditions which permitted decreases and increases in the urea of different portions of the same blood sample, the distribution remained the same as it was in the freshly drawn blood (Table I). Nor was it considered likely that a metabolic production of urea within the erythrocytes was the explanation. Such an action could lead to a constant absolute difference between the urea concentration in the cells and that in the plasma (a steady state in compliance with Frick's diffusion law), but high distribution ratios should then accompany low urea concentrations and *vice versa*. There was no such relation between the ratios and the urea values.

Two equations relating the distribution of urea between the cells and plasma to certain known and certain assumed factors were developed from Langmuir's adsorption isotherm,  $(1 - \theta)\alpha\mu = v\theta$ . The one, for appreciable adsorption, appeared to be not applicable, for it carried a term involving the urea concentration and it had been observed that the distribution was not related to the total urea present. However, that for but slight, or poor, adsorption reduced, in its simplest form, to  $R = 1 + (km/H_2O)$ , where  $R$  is the distribution ratio,  $k$  is a constant,  $m$  is the number of gm. of adsorbent in 100 ml. of cells, and  $H_2O$  is the volumes per cent of water in the cells. In developing this equation it was assumed that there was a

portion of the urea within the cells in osmotic equilibrium with the urea in the plasma and that there was another portion adsorbed on some material. Curiously, a very similar equation was derived from the mass action law and the assumption that some cell urea reversibly combined with some cell constituent in a bimolecular reaction. That equation was  $R = 1 + (kM/H_2O)$ , in which, as before, the symbols had the same significance, except that  $M$  represents the number of moles of non-urea reactant per 100 ml. of cells. Since neither of these equations involved the actual concentration of urea and since our ratios were not related to that actual concentration, it seemed that the underlying assumptions, that some urea was relatively fixed within the cell either by adsorption or by chemical union with a cell solute, were the most probable explanations of the unequal distribution of urea between the water of the cells and that of the plasma. Unfortunately, the analytical data afforded no means of deciding between those two possibilities.

Of all the cell constituents that might form a non-diffusing urea complex, hemoglobin is the most likely. The effect of urea on hemoglobin (49-53) and the behavior of heme with nitrogenous organic compounds (54-60) are well known. While very high concentrations of urea are necessary to produce a demonstrable denaturation of hemoglobin, it is possible that similar changes, to a lesser degree, might also occur at low urea concentrations. Moreover, it is possible that (though no claim is made that it does occur) urea might compete with globin for heme. Either action could easily account for our postabsorptive ratios, for, to do so, each gm. of hemoglobin would need to adsorb but 0.04 mg. of urea, or but 1 of every 23 molecules of hemoglobin (or 1 out of every 92 molecules of heme) would need to combine with just 1 of every 10 molecules of urea within the cells. In view of the above possibilities, it is our intention to investigate the "urea-binding" potentialities of hemoglobin and of heme.

We wish to thank E. R. Squibb and Sons for a generous supply of Squibb's Double Strength urease.

#### SUMMARY

1. An "arginase error" was not responsible for the results reported herein.

2. 66 blood samples from individuals in the postabsorptive state were analyzed. The individual and average figures are given for the urea nitrogen and the total water in the whole blood, in the plasma, and in the cells.

3. Urea is not equally divided between the water of the cells and that of the plasma. The distribution ratio is 1.14:1.00, on the average.

4. During the active production of urea after a meal, the ratio tends to be less than 1:1.

5. The possible factors involved in the unequal distribution of urea are discussed.

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# EFFECT OF TOCOPHEROLS ON PHOSPHORUS METABOLISM\*

BY LOUISE HARRIS WEISSBERGER AND PHILIP L. HARRIS

*(From the Departments of Biochemistry and Pharmacology and Radiology, School of Medicine and Dentistry, The University of Rochester, and the Laboratories of Distillation Products, Inc., Rochester, New York)*

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In this study the effects of normal, low, and high dietary intakes of vitamin E on the phosphorus turnover of various tissues of rats have been compared. Both tocopherol deficiency and overdosage caused a stimulation of phosphorus metabolism which statistical analysis showed to be highly significant.

## EXPERIMENTAL

Healthy young rats, weaned at 20 days and weighing between 40 to 50 gm., were used in these experiments. Litters were divided among three groups. In the early experiments, one group was fed ground dog chow (Purina) into which cottonseed oil (Wesson) had been thoroughly mixed at a 5 per cent level. A second group received this same diet except that a molecular distillate of vitamin E, containing 20 per cent of natural mixed tocopherols from vegetable oils, was substituted for the cottonseed oil. Consequently, these rats were receiving 100 mg. of tocopherol from each 10 gm. of diet consumed. The third group received a ration used in our vitamin E bioassays and known to be very deficient in tocopherols. It consisted of crude casein 20 per cent, corn-starch 27 per cent, cerelese 27 per cent, lard 12 per cent, salt mixture, U.S.P. No. 2, 4 per cent, and dried brewers' yeast 10 per cent. Vitamins A and D were supplied as a distilled fish oil concentrate and vitamin D<sub>2</sub>, respectively, to furnish 120 U.S.P. units of vitamin A and 50 U.S.P. units of vitamin D per 10 gm. of the diet.

In later experiments, the vitamin E-free diet was used for all of the animals and the tocopherol was administered separately by calibrated dropper. Four litter mate groups were employed. One received no tocopherol supplement, the second was fed 0.5 mg. of tocopherol per day, the third received 10.0 mg., and the fourth 100.0 mg. Consequently, the second group was comparable to the group fed the dog chow in the early experiments and the fourth group was similar to those on the earlier high vitamin E diet. The group receiving 10 mg. of tocopherols each day represented animals dosed with a moderate excess of vitamin E, approximately 10 to 100 times the minimum requirement.

\* Communication No. 48 from the Laboratories of Distillation Products, Inc., Rochester, New York.

These groups were maintained on their respective diets from weaning until 19 weeks of age. At various intervals during this time, small litter mate subgroups (except the 4 to 5 week-old groups, which were not litter mates) from each of the dietary régimes were given a solution of  $\text{Na}_2\text{HPO}_4$  ( $\text{P}^*$ , 20,000 to 75,000 counts per minute on our scale-of-four Geiger-Müller counters) by stomach tube and sacrificed 17 hours later. The tissues were removed, weighed, and prepared immediately for determination of activity. In all cases, samples of blood, one kidney, either both ovaries and uterine horns or one testicle, and the femur were taken. In a few instances, we removed the pelvis, the mandible (including teeth), and the tongue. In the case of the groups labeled Nos. 5 and 8a and of two dystrophic rats and their controls samples of striated muscle from various parts of the body were pooled.

The bone samples, from which the soft tissue had been removed, were dry-ashed and dissolved in 3 M HCl. The other tissues were dissolved in fuming nitric acid with the addition of superoxol when necessary. The radioactivity of the acid solutions was determined by the technique of Bale, Haven, and LeFevre (1).

Total phosphorus analyses (2) were carried out on enough samples of each tissue to warrant the conclusion that vitamin E did not affect the quantity of phosphorus per gm. of tissue of any of the age levels studied. The results are expressed as the per cent of the original dose per gm. of tissue, and the deviations and significance of the differences of the means were calculated by Fisher's method for small groups (3).

### Results

The results of the effect of vitamin E deficiency on the phosphorus turnover of various tissues of the rat are summarized in Table I. Table II shows the effect of a large excess of tocopherol and Table III, the effect of a moderate excess of tocopherol.

*Influence of Age on P Turnover*—As Chaikoff and his associates (4) and Falkenheim (5) have observed, phosphorus turnover is highest in the tissues of the young animal and falls off markedly during the growth period. The decrease in phosphorus turnover with increasing age pertains to all the tissues studied (femur, blood, kidney, and genitals) and must be borne in mind in the evaluation of the changes attributable to the tocopherol level of the diet. The values for a few very old rats suggest that the P metabolism, like the general metabolism, continues to decrease very gradually from the time of maturity throughout the life span of the animal.

*Vitamin E Deficiency*—The effect of vitamin E deficiency upon phosphorus metabolism is related to the age of the animal and to the duration of the deficiency. In the initial phase (up to 5 weeks), the lack of tocopherol

caused no change in phosphorus metabolism; in the intermediate phase (5 to 13 weeks), it resulted in an apparent, although statistically insignificant, acceleration of phosphorus turnover; eventually in prolonged vitamin E deficiency, a pronounced and significant stimulation of phosphorus metabolism was induced.

TABLE I  
*Effect of Vitamin E Deficiency on P Metabolism*

The figures in parentheses represent the number of animals in the group.

	Group, age in wks.	Dose of P* per gm tissue		Difference	SD, standard error of difference	p, probability
		Control mean	Experimental mean			
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Femur	4-5	7.65 (11)	5.50 (9)	-26.8	0.85	<0.01
	5	6.23 (6)	5.76 (6)	-7.5	0.98	
	8	2.53 (6)	3.27 (6)	+29.2	0.74	
	8a	2.04 (5)	2.30 (4)	+12.7	0.23	0.05
	13	1.09 (6)	1.28 (6)	+17.4	0.34	
	19	0.43 (4)	0.65 (4)	+51.2	0.08	
Kidney	4-5	1.43 (11)	0.85 (9)	-40.5	0.41	<0.01
	8	0.45 (5)	0.57 (6)	+26.7	0.14	<0.01
	13	0.51 (5)	0.52 (5)		0.14	
	19	0.21 (4)	0.39 (4)	+85.7	0.05	<0.01
Blood	4-5	0.33 (10)	0.24 (7)	-27.3	0.12	
	8	0.11 (6)	0.12 (6)		0.03	
	13	0.10 (6)	0.11 (6)		0.04	
	19	0.05 (4)	0.09 (4)	+76.5	0.02	0.02
Uterus and ovaries	4-5	0.40 (8)	0.15 (9)	-62.5	0.14	<0.01
	8	0.30 (3)	0.38 (3)	+26.7	0.06	
	13	0.24 (2)	0.28 (3)	+16.7	0.05	
	19	0.08 (2)	0.23 (2)	+173.8	0.01	
Testicle	5	0.47 (6)	0.38 (6)	-19.1	0.12	
	8	0.07 (3)	0.09 (3)	+28.6	0.01	
	8a	0.06 (5)	0.06 (4)		0.01	
	13	0.05 (3)	0.06 (3)	+13.2	0.02	
Muscle	19	0.04 (2)	0.06 (2)	+35.7	0.02	
	5	0.63 (6)	0.48 (6)	-23.8	0.16	
	8a	0.17 (5)	0.20 (5)	+17.6	0.02	0.1-0.05

In the young rat (4 to 5 and 5 week-old groups), vitamin E deficiency caused no increase in phosphorus turnover; in fact, the average experimental values are lower than the control figures, a change which is opposite in direction to that occurring in long term deficiency. The reality of this decrease is doubtful, since it is statistically insignificant in the 5 week-old group and in the 4 to 5 week-old group the controls were 4 days younger than the deficient animals.



Although an early trend toward higher values was apparent in the 8 and 13 week-old groups, a pronounced and significant increase in phosphorus metabolism occurred only in the 19 week-old group; *i.e.*, only after a prolonged period of vitamin E deficiency. Since the vitamin E-deficient rats showed a gain in weight equal to that of normal litter mates, this increase in phosphorus turnover was not attributable to a difference in growth and development. As Table I indicates, the effect of long term deficiency is reflected in all the tissues studied. Although too few determinations were made in the case of the genital tissues to warrant statistical analysis, the response in the testicle and uterus, as judged by the phosphorus turnover, was in all respects similar to that of the other tissues. The fact that the phosphorus turnover of the genital tissues showed no more sensitivity to tocopherol deficiency than that of the other tissues is of interest, since the most striking effect of vitamin E deficiency in the rat is reproductive failure. Furthermore, the effect of vitamin E deficiency on phosphorus metabolism was independent of the sex of the animal.

Two rats, 18 weeks of age, in which extremely severe symptoms of muscle dystrophy had developed as a result of vitamin E deprivation, were examined for comparison with their respective controls. The phosphorus turnover of the skeletal muscle, femur, and blood of the dystrophic rats was approximately twice that of the controls, while the phosphorus content of these tissues was normal. Since the reported morphological and metabolic abnormalities characteristic of dystrophy are largely limited to the skeletal muscle, it is of interest that in these dystrophic rats the acceleration of the phosphorus metabolism of the bone and blood was approximately equal to that of the muscle.

*Effect of High Vitamin E Intake*—An excessive intake of vitamin E, 100 mg. of tocopherol per day, which was estimated as 100 to 1000 times the daily requirement, resulted in a striking stimulation of phosphorus turnover at all the age levels studied. This acceleration showed up most consistently and to the greatest extent in the femur in which the difference in turnover was usually highly significant ( $p < 0.01$ ). An increase in phosphorus turnover, of the same order of magnitude, was also observed in the pelvis and mandibles of rats of the 4 to 5 and 17 week-old groups on the high vitamin E régime. This stimulating effect of excess vitamin E, therefore, probably pertains to the hard tissues as a whole and is not limited to the long bones.

Although the response of the soft tissues (kidney, blood, genitals, and muscle) to excess vitamin E was in the same direction as that described for the femur, it was far less uniform and less marked. As Table II shows, the experimental values frequently, but not always, exceed the control values. In some groups the difference is significant, while in others it is not; *e.g.*,

the phosphorus turnover of the kidney was significantly increased by excess vitamin E at 8, 17, and 19 weeks, while at 4 to 5 and 13 weeks the control and experimental values were practically identical. In no tissue

TABLE II

*Effect of High Vitamin E Intake on P Metabolism*

100 mg. of tocopherols were given per day. The figures in parentheses represent the number of animals in the group.

	Group, age in wks	Dose of P* per gm tissue		Difference	SD, standard error of difference	p, probability
		Control mean	Experimental mean			
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Femur	4-5	7.65 (11)	10.30 (2)	+34.6	0.85	
	5	6.23 (6)	9.57 (6)	+53.6	1.01	<0.01
	8	2.53 (6)	4.03 (6)	+59.3	0.71	<0.01
	8a	2.04 (5)	3.68 (5)	+80.4	0.48	<0.01
	13	1.09 (6)	1.46 (6)	+33.9	0.36	
	17	0.82 (2)	1.22 (4)	+48.8	0.07	<0.01
	19	0.43 (4)	0.82 (5)	+90.7	0.01	<0.01
Kidney	4-5	1.43 (11)	1.63 (2)	+14.0	0.65	
	8	0.45 (5)	0.71 (6)	+57.8	0.14	<0.01
	13	0.51 (5)	0.50 (5)		0.17	
	17	0.41 (2)	0.58 (4)	+41.5	0.07	0.02-0.01
	19	0.21 (4)	0.42 (5)	+100.0	0.05	<0.01
Blood	4-5	0.33 (10)	0.34 (2)		0.08	
	8	0.11 (6)	0.19 (6)	+72.7	0.02	<0.01
	13	0.10 (6)	0.12 (6)		0.04	
	17	0.11 (1)	0.12 (4)		0.03	
	19	0.05 (4)	0.09 (4)	+70.6	0.01	<0.01
Uterus and ovaries	4-5	0.40 (8)	0.68 (2)	+70.0	0.17	
	8	0.30 (3)	0.39 (3)	+30.0	0.04	0.05-0.02
	13	0.24 (2)	0.29 (3)	+20.8	0.05	
	17	0.25 (2)	0.40 (4)	+60.0	0.11	
	19	0.08 (2)	0.22 (2)	+161.9	0.04	
Testicle	5	0.47 (6)	0.84 (6)	+78.7	0.22	
	8	0.07 (2)	0.09 (3)	+32.8	0.03	
	8a	0.06 (5)	0.13 (5)	+100.0	0.02	<0.01
	13	0.05 (3)	0.07 (3)	+30.2	0.02	
	19	0.04 (2)	0.05 (3)	+19.0	0.01	
Muscle	5	0.63 (6)	0.75 (6)	+19.0	0.21	
	8a	0.17 (5)	0.29 (4)	+67.6	0.05	0.01

except the femur did excess vitamin E cause an increase in phosphorus turnover at all the age levels, nor did it cause a significant increase in phosphorus turnover of all the tissues at any one age level. Neither could any correlation be made between the response of the soft tissues to excess tocopherol and the sex of the animal.

The body weights of the rats fed the high level of tocopherol were comparable with those of the control animals, as shown by the average weights of the two groups at the 19 week period: control, 313 gm. for the males and 212 gm. for the females; high vitamin E, 310 gm. for the males and 203 gm. for the females. Consequently, at all age levels studied, we were dealing with animals of the same age and size.

*Effect of Moderately High Intake of Vitamin E*—Since this stimulation of phosphorus metabolism was produced by an intake of vitamin E which was 100 to 1000 times the requirement, it was of interest to determine the effect of a moderately high vitamin E intake on phosphorus turnover. For this purpose, 10 mg. of tocopherol per day were fed to litter mates of Groups 5 and 8a as a relative approximation of the level of tocopherol which might

TABLE III

*Effect of Moderately High Vitamin E Intake on P Metabolism*

10 mg. of tocopherols were given per day. The figures in parentheses represent the number of animals in the group.

	Group, age in wks.	Dose of P* per gm. tissue		Difference	SD, standard error of difference
		Control mean	Experimental mean		
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Femur	5	6.23 (6)	7.17 (6)	+15.1	0.75
	8a	2.04 (5)	1.92 (4)	-5.9	0.24
Testicle	5	0.47 (6)	0.51 (5)	+8.5	0.15
	8a	0.06 (5)	0.06 (5)		0.01
Muscle	5	0.63 (6)	0.66 (6)	+4.8	0.16
	8a	0.17 (5)	0.18 (5)		0.04

be used clinically. As Table III shows, vitamin E at this level had no influence on phosphorus turnover except for the femur turnover in the 5 week-old group. In that case, the increase of 15 per cent, following 10 mg. of tocopherol per day, was small in comparison to the increase of 54 per cent obtained by feeding 100 mg. of tocopherol per day to litter mates.

This phenomenon of an elevation in phosphorus turnover thus resulted from an extremely high vitamin E intake and was not produced by a moderate excess.

## DISCUSSION

Our observation that prolonged vitamin E deficiency results in an acceleration of phosphorus metabolism is consistent with the metabolic abnormalities which have been shown to occur in dystrophic muscle. In muscular dystrophy, the oxygen consumption (6-8) and succinoxidase ac-

tivity (9) of the skeletal muscle are elevated. Since oxidation and phosphorylation are coupled in muscle, an increase in phosphorus turnover should parallel an increase in the rate of the oxidative processes. In our dystrophic rats, we found an increase in phosphorus turnover of the same order of magnitude as the increase in oxygen consumption reported by Houchin and Mattill for similarly treated rats (100 to 125 per cent) (7). Kaunitz and Pappenheimer (8) have shown that high oxygen consumption of muscle in vitamin E deficiency precedes the morphological changes; similarly, in our 19 week-old group of vitamin E-deficient, non-dystrophic rats, the increase in phosphorus turnover was evident, showing that the acceleration of phosphorus metabolism also precedes the clinical syndrome.

Houchin (9) has suggested that in normal muscle oxidation vitamin E acts as an inhibitor upon the succinoxidase system. In vitamin E deficiency the oxygen consumption is elevated due to lack of this inhibitor. From this point of view, the observed increase in phosphorus turnover in tocopherol deficiency is not a stimulation of phosphorus metabolism in the strict sense, but rather the result of a lack of the oxidation inhibitor, vitamin E.

The low creatine content of dystrophic muscle and accompanying creatinuria (7, 10) also suggest that an elevated phosphorus metabolism occurs in muscular dystrophy. Creatine as phosphocreatine is a link in the muscle phosphate cycle. An excessive and continuous loss of creatine from the muscle would impair the resynthesis of phosphocreatine and, therefore, the transfer of phosphate in the carbohydrate cycle. The resultant decrease in the efficiency of the utilization of phosphate would cause an increase in the rate of phosphate turnover. In the presence of marked phosphorus, this showed up as an increase in the  $P^*:P$  ratio of the muscle.

In the absence of any change in phosphorus content, an increase in the rate of phosphorylation of one tissue will eventually be reflected in the phosphorus turnover of other tissues. Thus, there are two possible interpretations of our finding that prolonged vitamin E deficiency causes an increase in the phosphorus turnover of the bone and soft tissues. (1) The lack of tocopherol may exert a general effect in raising the phosphorus metabolism, in which case the bone and soft tissue changes would be the direct result of the deficiency. (2) The primary effect may be limited to the muscle and the increased phosphorus turnover observed in the other tissues would be a reflection of the increased muscle turnover. Our data furnish no clue as to the primary and secondary nature of the elevation of the phosphorus metabolism in the bone and soft tissues. However, the second interpretation can be supported by the report of Kaunitz and Pappenheimer (8). They present some evidence to show that the elevation in oxygen consumption is limited to muscle, for the livers of their vitamin

E-deficient animals did not show an increased  $QO_2$  and the elevation in basal metabolic rate could be accounted for by the increased oxidation in muscle.

The fact that both prolonged vitamin E deprivation and overdosage caused a shift in P metabolism in the same direction appeared at first to be paradoxical. It is evident, however, that the two conditions are quite distinct in regard to their influence on P turnover: (1) In the bone, at least, a large excess of tocopherol caused a significant increase in turnover at all the ages studied, while lack of the vitamin resulted in a significant increase only in the oldest rats, the animals which had been on the deficient diet for the longest time. (2) There is a quantitative difference in the two pictures. In the femur, where the largest and most consistent increases occurred, the P turnover in animals on the high vitamin E diet always exceeded the value for the vitamin E-deficient rats. In the soft tissues, the difference is less marked, but there is a trend in the same direction. (3) No muscular lesions were found in animals which had received a large excess of tocopherols for long periods of time. On the other hand, a rarefaction of the long bones has been observed<sup>1</sup> under these conditions. This effect on P metabolism seems to be limited to very high overdosage, at least 100 times the estimated daily requirement, for animals on a moderately high tocopherol intake showed no increase in P turnover.

Until further work is done to elucidate the metabolic effects of vitamin E deficiency and overdosage, we can only conclude that both conditions bring about an over-all increase in phosphorus turnover. The effects of the two conditions on phosphorus metabolism are similar, but not identical, and presumably the underlying mechanisms are not the same.

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#### SUMMARY

1. The phosphorus turnover of the bone, kidney, blood, and genitals of rats of various ages on low vitamin E, high vitamin E, and normal diets has been compared.

2. Prolonged tocopherol deficiency resulted in marked elevation of phosphorus turnover in the bone and soft tissues at 19 weeks of age; a trend in this direction was apparent at 8 and 13 weeks.

3. Excessive vitamin intake, 100 mg. of natural mixed tocopherols per rat per day, caused an increase in the phosphorus metabolism in the bone

<sup>1</sup> Harris, P. L., and Joffe, M., unpublished data.

at all ages studied. Sometimes the phosphorus turnover in the soft tissues was elevated; sometimes this did not occur.

4. A moderately high vitamin E intake, 10 mg. of tocopherols per day, brought about no change in phosphorus metabolism.

5. Vitamin E, under our experimental conditions, had no effect upon the P content of the tissues nor was there any apparent sex difference in response.

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# THE KINETICS OF THE ENZYME-SUBSTRATE COMPOUND OF PEROXIDASE

By BRITTON CHANCE

(From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, and the Physiological Laboratory, University of Cambridge, Cambridge, England)

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Studies on the over-all kinetics of enzyme action revealed in the majority of cases and over certain concentration ranges that the enzymatic activity was related linearly to the enzyme concentration and hyperbolically to the substrate concentration. On the basis of such evidence Michaelis and Menten (13) showed that such relationships were explained on the assumption that an intermediate compound of enzyme and substrate was formed:  $E + S \rightarrow ES \rightarrow E + P$ . As the rate of formation of such a compound was assumed to be quite rapid, the rate of breakdown was the rate-determining step. This theory was extended by Briggs and Haldane (2) who pointed out that the rate of formation of the intermediate compound could in certain cases be limited by the number of collisions of enzyme and substrate, and modified the Michaelis theory accordingly. The resulting theory has been extremely useful as a first approximation in the explanation of enzyme action and has given a basis for the comparison of different enzymes in terms of their affinity and activity.

The reaction velocity constants are, however, lumped into one term, the Michaelis constant, and are not separately determined. It is the purpose of this research to determine these constants separately, and to show whether the Michaelis theory is an adequate explanation of enzyme mechanism. Moreover, studies on the over-all enzyme activity do not permit a determination of whether the enzyme-substrate compound exists in fact and, if it exists, whether such a compound is responsible for the enzyme activity.

Several attempts have been made to identify enzyme-substrate compounds. Stern (16) made direct spectroscopic measurements of the compound of catalase and ethyl hydroperoxide and found that this compound was unstable and decomposed after several minutes in the presence of 1 M ethyl hydroperoxide. This was interpreted to indicate that the intermediate compound was responsible for the decomposition of all the ethyl hydroperoxide in this period. Although independent tests showed that ethyl hydroperoxide was decomposed by catalase, no data were given on the amount or rate of decomposition of ethyl hydroperoxide in the spectroscopic experiment (Green (8)).



Keilin and Mann (11) studied the compound of peroxidase and hydrogen peroxide by visual spectroscopy. Their observations include the fact that a spectroscopically defined compound of peroxidase and hydrogen peroxide is formed and that this compound rapidly decomposes in the presence of an oxygen acceptor. While these experiments indicate the existence of an unstable intermediate compound, no direct relation between this intermediate compound and the enzymatic activity is given. A conclusive proof of the Michaelis theory rests on such evidence.

This paper describes a detailed study of the compound of horseradish peroxidase and hydrogen peroxide, an enzyme-substrate compound. The enzyme activity in the presence of leucomalachite green, an acceptor, and hydrogen peroxide, a substrate, has been studied in the usual manner and the Michaelis constant determined. A new apparatus and a new method of studying the kinetics of rapid reactions have been developed and used to measure directly the reaction velocity constants which compose the Michaelis constant. These are the rates of formation and breakdown of the enzyme-substrate compound. The equilibrium of enzyme and substrate in the absence of an acceptor has also been studied. These new data have then been compared with the Michaelis constant which has been determined in the classical manner. A point by point comparison between experiment and theory has been made possible by solutions of the differential equations representing the Briggs and Haldane modifications of the Michaelis theory. In this way, the validity of the Michaelis theory has been clearly demonstrated, and the important relationship between the enzyme-substrate compound and its activity has been clearly shown. A preliminary report of this work was given earlier (Chance (4)).

*Preparation and Standardization*—The method of Elliott and Keilin (7) was used for the preparation of peroxidase. The first alcohol precipitate was usually discarded and in a particular case 1 gm. of enzyme,  $PZ^1 = 256$ , was obtained from 7 kilos of horseradish. The enzyme was kept in a volume of 75 cc. and was tested periodically for hematin iron and  $PZ$ . As neither the apparatus nor the information was available at the time, the peroxidase was not purified further in the manner recently indicated by Theorell (18).

A typical preparation contained  $5 \times 10^{-5}$  M hematin iron. The light absorption was measured at 640 and 400  $m\mu$  with a grating photoelectric spectrophotometer and it was found that  $\epsilon_{640} = 12 \pm 2$  and  $\epsilon_{410} = 125 \pm 12$

<sup>1</sup>  $PZ$  or purpurogallin number indicates peroxidase activity in terms of mg. of purpurogallin formed from pyrogallol in 5 minutes at 20° per mg. of dry weight of enzyme preparation. 12.5 mg. of  $H_2O_2$  and 1.25 gm. of pyrogallol in 500 cc. of water are used.

( $c = 1$  mm,  $d = 1$  cm.) at pH 6.2 in 0.01 M phosphate buffer on the basis of total hematin iron.<sup>2</sup> The extinction coefficients given do not represent those of a pure peroxidase.

Perhydrol, diluted to 1 M and kept at 0°, was tested periodically by permanganate titration. Further dilutions were freshly made up before each experiment.

A slightly oxidized saturated solution of leucomalachite green in 0.05 M acetic acid was standardized by oxidation in the presence of peroxidase and hydrogen peroxide. The light absorption at 610 m $\mu$  was measured and the concentration determined in terms of a standard solution of malachite green ( $\epsilon_{614} \doteq 50$ ). The pH was maintained by 0.05 M acetate buffer at 4.1.

### Method

This is set forth elsewhere (Chance (3, 5, 6)). The Hartridge-Roughton (10) flow method has been modified to give fluid economy and photoelectric resolution greatly exceeding the designs of Roughton and Millikan (15) and adequate for the direct measurement of the kinetics of the hematin compounds in a 1 mm. bore observation tube at concentrations of  $1 \times 10^{-6}$  mole of hematin Fe per liter. The apparatus is shown in Fig. 1, and details of the various parts may be obtained in the references above.

*Controls*—Detailed controls on the efficient mixing by this apparatus have been described in a previous paper (Chance (3)), indicating that the mixing was essentially complete in  $2 \times 10^{-4}$  second for the highest values of flow velocity. In these experiments the times were long compared to the minimum time range of the apparatus.

Controls on the linearity of the photoelectric system were carried out by plotting deflection of the recorder against concentration of the reactant and a linear relationship was obtained, as the light absorption was very small.

Under certain conditions, the production of malachite green may interfere with the measurement of the kinetics of the intermediate compound. The absorption of the dye is rather high at 420 m $\mu$ , as shown in Fig. 2, and would add to the absorption of the enzyme. A  $4 \times 10^{-6}$  M malachite green solution would cause a 3 per cent error in the measurement of  $1 \times 10^{-6}$  M hematin Fe peroxidase solution. This sets a limit to the amount of malachite green formed in the presence of a given amount of enzyme.

A compensation for the effect of malachite green absorption was effected by varying the relative amounts of light incident on the 370 and 430 m $\mu$  filter combinations so that the absorption of malachite green affected each photocell equally.

$$^2 \epsilon \text{ (extinction coefficient)} = \frac{\log_{10} I_0/I}{d \text{ (cm.)} \times c \text{ (mm per liter)}}$$

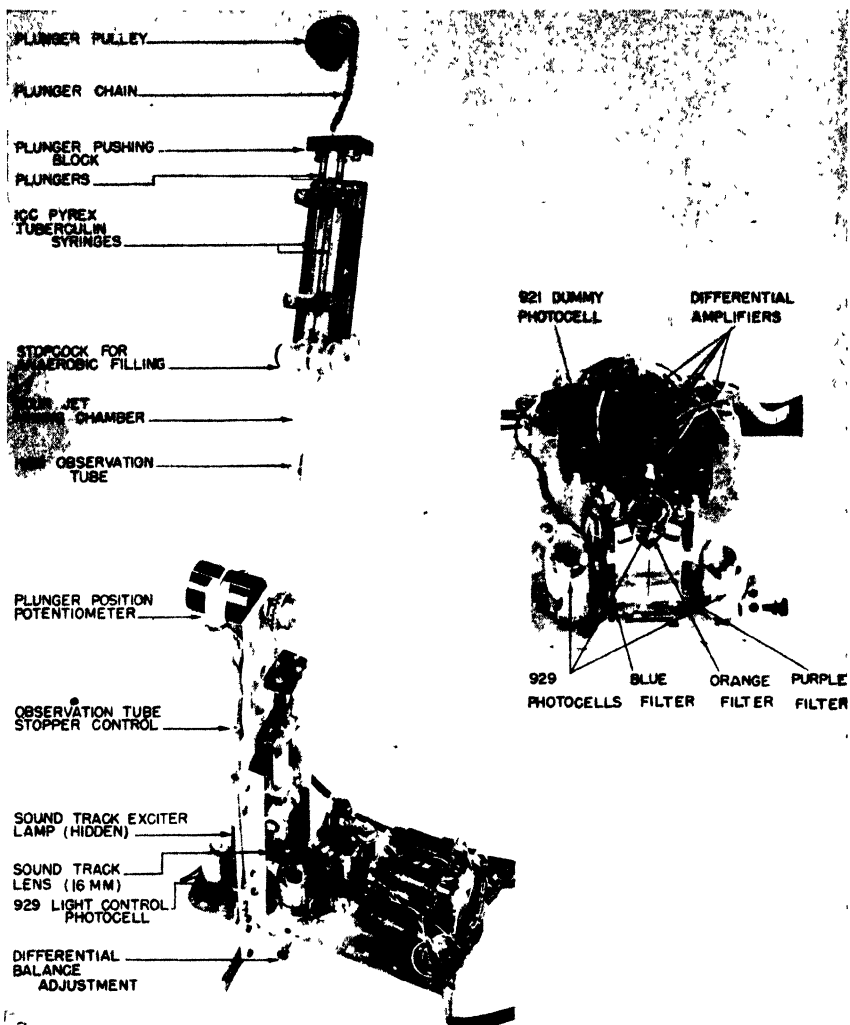


FIG 1 Syringe unit, photocell unit, and assembled apparatus Light and electrostatic shields are removed.

### *Procedure*

In order to explain the experimental method more clearly the procedure used to obtain the data of Fig. 3 will be outlined. The enzyme solution was centrifuged before experiment in order to remove denatured protein and give a clear brown solution. Shortly before an experiment, the enzyme

was diluted to  $2 \times 10^{-6}$  M hematin Fe. Hydrogen peroxide was diluted to  $16 \times 10^{-6}$  M just previous to an experiment. A saturated solution of leucomalachite green in 0.05 M acetic acid was diluted to  $60 \times 10^{-6}$  M in acetate buffer to make the final pH 4.0.

The syringes shown in Fig. 1 were thoroughly rinsed with cleaning solution and carefully flushed out with water in order that there might be no trace

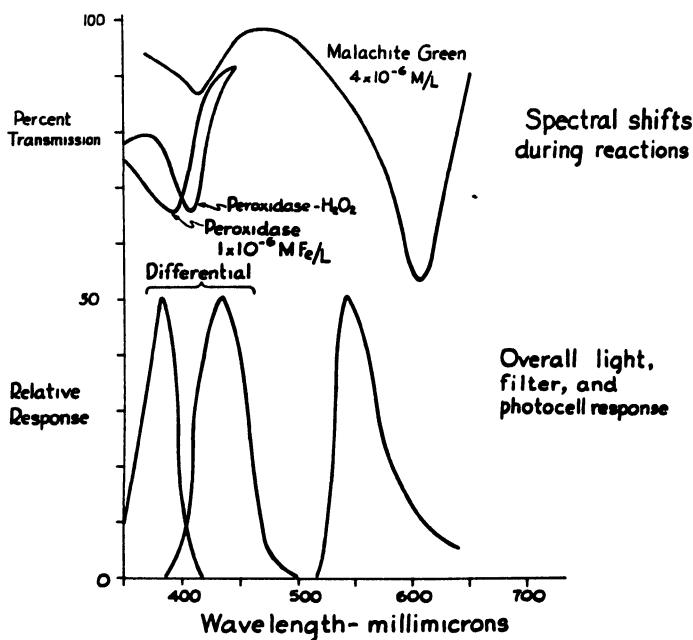


FIG 2 The upper curves give the light transmission of enzyme, enzyme-substrate compound, and oxidized acceptor under the conditions of Fig. 3. The lower curves give the filter combinations used to measure the kinetics of the reactions. The trough depth was 16 times that of the 1 mm observation tube of the rapid reaction apparatus. The spectral interval was approximately  $8 \text{ m}\mu$ . The wave-length markers read  $15 \text{ m}\mu$  low.

of the enzyme in the tube which was to be filled with substrate and acceptor. The right-hand syringe was then filled with a mixture of  $8 \times 10^{-6}$  M hydrogen peroxide and  $30 \times 10^{-6}$  M leucomalachite green in 0.05 M acetate buffer. These reactants were squirted into the top of the syringe while the outlet was held closed with a small rubber pad mounted on a lever shown in Fig. 1. The syringe plunger was then entered in the barrel and held in place at the top of the syringe by means of a plunger driving block. The left-hand syringe was flushed out with water and filled with  $2 \times 10^{-6}$  M

enzyme solution while the outlet tube was again held closed by means of the stopper. The plunger for the left syringe was then entered and fitted into the driving block. Both plungers were carefully pushed a few mm. down their respective barrels to make sure that they were running smoothly and were accurately aligned. The zero point of the recording mirror oscillograph was checked and a trial run was made by sharply pushing the driving block approximately 1 cm. This caused the reactants to be mixed and to flow down the observation tube very rapidly and, at the end of the

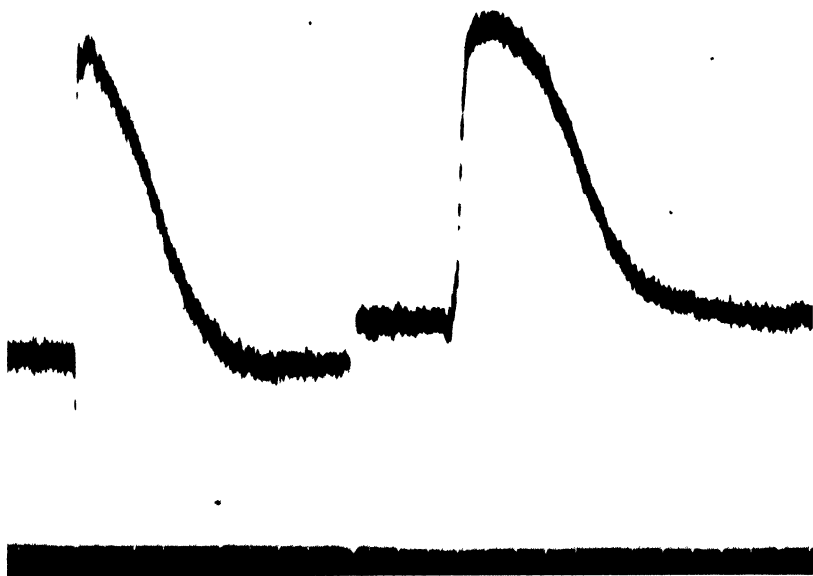


FIG. 3. Mirror oscillograph recording of the production of malachite green (left) and the corresponding kinetics of the enzyme-substrate compound (right). Time markers, 0.2 second. Peroxidase =  $1 \times 10^{-6}$  mole of hematin Fe per liter,  $\text{H}_2\text{O}_2 = 4 \times 10^{-6}$  mole per liter, leucomalachite green =  $15 \times 10^{-6}$  mole per liter, pH = 4.0.

discharge, to stop before the photocell and light beam. The progress of the reaction that ensued in the portion of liquid stopped in the path of the light beam was measured directly by the photoelectric amplifiers. Either Amplifier 1 or 2 could be used, as shown in Chance (5). If the deflection was too large, the amplifier gain was readjusted so that the picture was approximately three-quarters of full linear scale. If it was then considered that the experiment was suitable for recording, the camera attached to the mirror oscillograph was set in operation, and the syringe plungers were given a second sharp push which caused the kinetic curves to repeat themselves. In this way the kinetics of the intermediate compound and the over-all

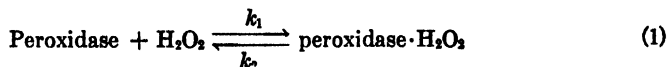
reaction were recorded. This process was repeated until the syringes were completely discharged, and in most cases it was found that three to six curves could be obtained from one filling of the syringes.

A second experiment was carried out immediately to calibrate the maximum concentration of the enzyme-substrate compound. This was done in the same manner as the first experiment except that the leucomalachite green was omitted. Hence the substrate concentration would be sufficient to saturate the enzyme completely, as was indicated by independent experiment. This reaction was also recorded photographically. The deflection corresponded to  $1 \times 10^{-6}$  M hematin Fe enzyme-substrate compound and is marked on Fig. 10.

A third experiment was necessary to calibrate the amount of malachite green formed. Malachite green, formed by peroxidase action, was diluted to  $4 \times 10^{-6}$  mole per liter and used to calibrate the photoelectric amplifier of the system measuring the rapid reaction. The right-hand syringe was filled with the malachite green solution, and the left-hand syringe was filled with water. These two solutions were pushed down, not simultaneously, but alternately, so that the observation tube was filled first with malachite green and then water. The resulting deflection was recorded photographically and gave the deflection corresponding to  $4 \times 10^{-6}$  M malachite green. In this way, the amount of malachite green which had been formed in the experiment was accurately determined. This calibration point appears in Fig. 10. These calibrations were made so that it was unnecessary to rely upon any long time stability of the photoelectric amplifier or recording system.

### Results

#### *Equilibrium of Enzyme and Substrate*



This reaction was studied by direct photoelectric measurements of the equilibrium concentration of enzyme-substrate compound as a function of substrate concentration. If hydrogen peroxide is mixed with peroxidase, the spectrum changes as in Fig. 2 and the compound denoted peroxidase- $\text{H}_2\text{O}_2$ , Complex I (Keilin and Mann (11)), is formed, as the substrate is not in great excess.

In order to measure this equilibrium it is essential that  $k_3$ , the first order velocity constant for the enzymatic breakdown of the intermediate compound, be negligible compared to  $k_1$ , the second order constant for the combination of enzyme and substrate, and  $k_2$ , the first order constant for the reversible breakdown of the enzyme-substrate compound. As Keilin has

pointed out, the small amount of acceptor present in the enzyme preparation may be oxidized by the addition of hydrogen peroxide and under these conditions the enzymatic breakdown of the enzyme-substrate compound is small. Under these conditions the intermediate compound appeared moderately stable at pH 6.2, although its concentration remained constant for only 5 to 10 seconds at pH 4.2. However, complete stability was not essential for measurements in the rapid reaction apparatus, and it was desired to carry out these reactions at the same pH as the other studies (4.0).

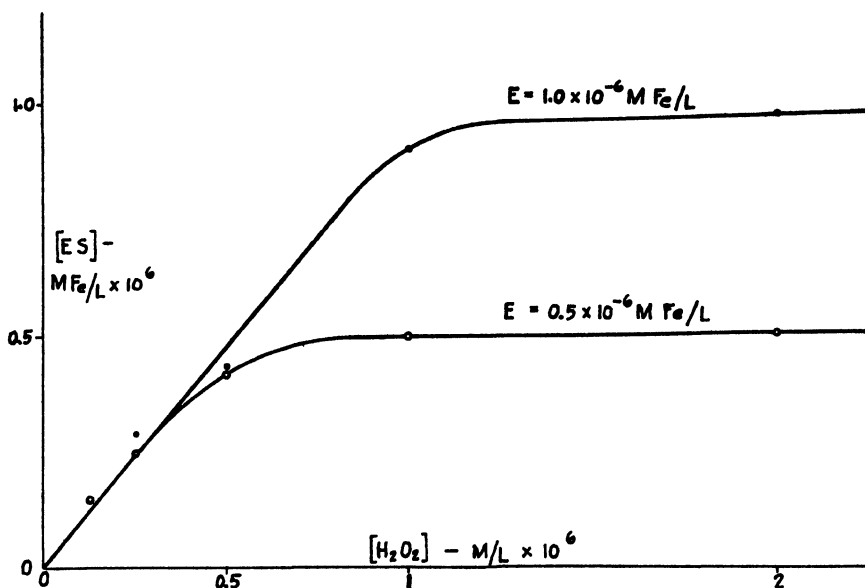


FIG. 4. Equilibrium of enzyme and substrate in absence of acceptor. Ordinate, intermediate compound as total hematin iron; abscissa, initial  $H_2O_2$ . pH = 4.2.

The experiments were carried out in this manner. The left-hand syringe was filled with varying concentrations of substrate, while the right-hand syringe was filled with a known concentration of enzyme. Both syringe plungers were then pushed downward in short, sharp pushes so that the observation tube was filled with mixed but unchanged enzyme and substrate, and, after the flow had stopped, the photoelectric system measured and recorded the rate of formation of the intermediate compound and the equilibrium concentration of enzyme-substrate compound. This experiment was repeated for different initial substrate concentrations, and the equilibrium value of the enzyme-substrate compound is plotted in Fig. 4 against initial substrate concentration. It is assumed that the maximum ordinate

corresponds to complete conversion of enzyme into enzyme-substrate compound of concentration equal to the independently determined molar hematin iron.

The data of Fig. 4 indicate very small dissociation of the intermediate compound, and the equilibrium constant estimated from two points on Fig. 4 giving finite values is  $2 \times 10^{-8}$ . As the enzymatic breakdown of the enzyme-substrate compound was not zero, this figure should be regarded as a minimum value. Evidently the enzyme was nearly completely converted into its enzyme-substrate compound by an equimolal concentration of substrate. This indicates that all this hematin iron existed as compounds capable of reacting similarly with hydrogen peroxide, *i.e.* forming a spectroscopically defined intermediate compound.

*Rate of Formation of Enzyme-Substrate Compound*



The rate of this reaction has been determined in the manner described before; namely, the right-hand syringe is filled with a  $2 \times 10^{-6}$  M hydrogen peroxide solution, while the left-hand syringe is filled with a  $2 \times 10^{-6}$  M hematin iron enzyme solution. The syringe plungers are again pushed down rapidly, and the reaction was measured after the flow had stopped in the observation tube. The half time of this reaction was 0.1 second. The experiment was then repeated with substrate concentrations from 0.5 to  $8 \times 10^{-6}$  M. The half time and curve shapes of these data were measured, and it was found that a bimolecular equation approximately satisfied the variation of rate with substrate concentration. Higher substrate concentrations have not been used to a great extent, as there is some question whether or not a compound of different spectral absorption denoted peroxidase-H<sub>2</sub>O<sub>2</sub>, Complex II (Keilin and Mann (11)), might be formed. There is also slight evidence to lead one to believe that the reaction might not follow a bimolecular course at substrate concentrations greater than  $10 \times 10^{-6}$  mole per liter. Experiments in which concentrations of substrate lower than  $0.5 \times 10^{-6}$  mole per liter are employed involved larger experimental errors, owing to the small changes in light transmission.

The data fit a second order kinetic equation, as Fig. 5 shows. Over a range of enzyme concentrations from 1 to  $2 \times 10^{-6}$  mole of hematin Fe per liter and a range of substrate concentrations from 0.5 to  $4 \times 10^{-6}$  mole per liter the mean value of the second order velocity constant was  $1.2 \times 10^7$  liter mole<sup>-1</sup> sec.<sup>-1</sup>. The mean error is  $0.4 \times 10^7$ . The previous section gave the ratio of  $k_2$  to  $k_1$  as  $2 \times 10^{-8}$ , or larger; hence  $k_2$  is 0.2 sec.<sup>-1</sup> or less.

It is now apparent that the enzyme and substrate unite with extreme



rapidity to form a relatively tight complex, and it is interesting to note that the ratio of  $k_2/k_1$  is considerably smaller than the Michaelis constant determined by measurement of the over-all enzyme action ( $5 \times 10^{-6}$ , Mann (12)).  $k_3$  is possibly far greater than  $k_2$  in the case of peroxidase, and this will be shown to be true in the next section.

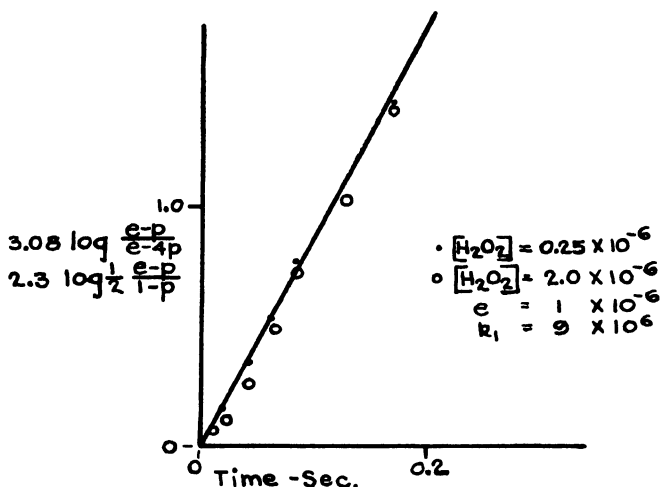
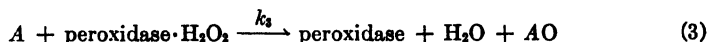


Fig. 5. Kinetics of formation of intermediate compound plotted for two values of substrate concentration according to the second order equation.  $k_1 = 9 \times 10^6$  liter mole<sup>-1</sup> sec.<sup>-1</sup>, pH = 4.0.

#### *Rate of Breakdown of Enzyme-Substrate Compound*



The decomposition of the intermediate compound in the presence of an oxygen acceptor is shown schematically by Equation 3. We will choose an oxygen acceptor in the presence of which peroxidase has a high activity. The oxidation products must not interfere with the measurement of the enzyme-substrate compound. This restriction eliminates acceptors like pyrogallol, hydroquinone, and guiacol, while leucomalachite green and ascorbic acid were found to be most satisfactory. In order to demonstrate the effect of such oxygen acceptors on the enzyme-substrate compound, the enzyme is mixed with substrate and acceptor, and the kinetics of the intermediate compound are observed. In Fig. 6 the concentration of the intermediate compound is recorded as a function of time for various concentrations of ascorbic acid. (In contrast to the results of Tauber (17) a polyphenol was not essential in this process.) The right-hand syringe is

filled with a mixture containing  $8 \times 10^{-6}$  M  $\text{H}_2\text{O}_2$ , 0.05 M acetate buffer, pH 4.2, and varying concentrations of ascorbic acid. The left-hand syringe is filled with  $2 \times 10^{-6}$  M enzyme solution. The curves show that in the presence of  $2.9 \times 10^{-6}$  mole of ascorbic acid, the intermediate compound is stable for a long period of time. The stability of the compound is indicated, of course, by the length of time required for its concentration to fall to zero, for this is taken to mean that all the substrate has been con-

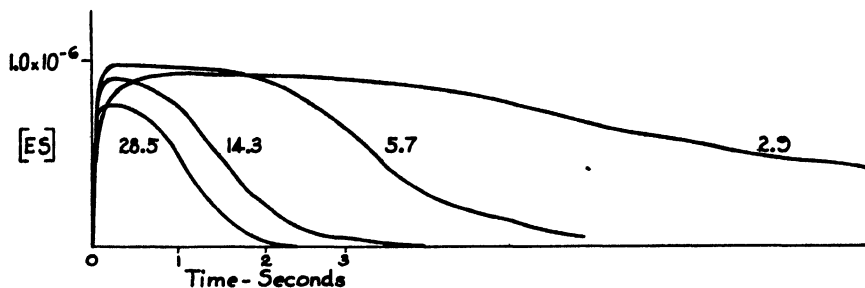


FIG. 6. The effect of an acceptor on the kinetics of the enzyme-substrate compound.  $E = 1 \times 10^{-6}$  mole of hematin Fe per liter,  $\text{H}_2\text{O}_2 = 4 \times 10^{-6}$  mole per liter, ascorbic acid as indicated in micromoles per liter, pH = 4.2.

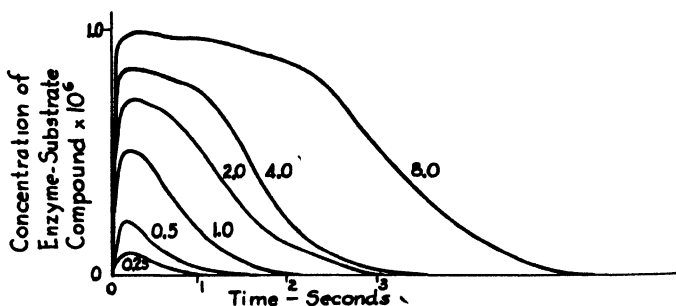


FIG. 7. The effect of substrate on the kinetics of the enzyme-substrate compound.  $E = 1 \times 10^{-6}$  mole of hematin Fe per liter, ascorbic acid approximately  $14 \times 10^{-6}$  mole per liter, initial  $\text{H}_2\text{O}_2$  as indicated in micromoles per liter, pH = 4.2.

sumed. The curves of Fig. 6 for higher concentrations of ascorbic acid clearly show a marked decrease in this interval. The curves also indicate a decrease in the maximum concentration of the enzyme-substrate compound,  $p_{\max}$ , with increasing ascorbic acid concentration. This decrease in  $p_{\max}$  is due to the higher rate of breakdown of the intermediate compound. The low value of  $p_{\max}$  in the  $2.9 \times 10^{-6}$  M ascorbic acid curve is believed due to experimental error.

The effect of the substrate concentration is shown in Fig. 7, when the acceptor concentration has been maintained in excess of the substrate con-

centration. The first interesting feature of this family of curves is the variation of height of the curves with substrate concentration, giving a method of directly studying enzyme-substrate affinity from measurements of the enzyme-substrate compound rather than from the over-all enzyme action. It is seen, for the particular value of ascorbic acid concentration, that the enzyme is one-half saturated by  $1 \times 10^{-6}$  M initial substrate concentration. It should also be noted that the area under each curve increases regularly with the initial substrate concentration. One would expect this, as  $k_3$ , the rate of breakdown of the enzyme-substrate compound, should be constant as the acceptor concentration is maintained constant and it is found that the area under the curve is proportional to the total amount of hydrogen peroxide consumed.

While  $k_3$  can be determined from the kinetics shown above, we have yet to devise an experiment in which the rate of breakdown of the intermediate compound is determined from both enzyme-substrate kinetics and the rate of production of oxidized acceptor. This experiment is of great importance in determining the relation between the over-all reaction and the kinetics of the enzyme-substrate compound. The rate of disappearance of ascorbic acid could not be measured with this apparatus, as it was not adaptable for wave-lengths below 350 m $\mu$ . Leucomalachite green was used as an oxygen acceptor for the following reasons. (1) The mechanism of its oxidation appears simple compared to that of pyrogallol. (2) The absorption is quite strong and does not seriously interfere with the measurement of the enzyme absorption. (3) The linearity between enzyme concentration and rate of formation of malachite green is quite good.

One experimental difficulty in the use of leucomalachite green is a variation in the amount of the dye formed. Only when the leuco base is partially oxidized is the full amount realized and not even then at higher enzyme concentrations. This phenomenon is not completely understood.

On the right-hand side of Fig. 3 are shown the kinetics of the intermediate compound recorded by a photokymograph. The time is read from left to right with markers every 0.2 second. The break in the base-line corresponds to the moment when the syringe plungers were pushed downwards and, after 0.1 second, the flow stops and the reaction of enzyme, substrate, and acceptor proceeds. The formation of the intermediate compound occurs quite rapidly, as is indicated by the abrupt upward deflection of the tracing. Within 0.1 second the enzyme-substrate compound has reached its maximum concentration ( $p_{\max.}$ ), and it maintains a steady state for 0.2 second. After this time the substrate concentration has fallen to such a value that the rate of formation of the intermediate compound no longer balances its rate of breakdown. Hence its concentration decreases rapidly and in 1 second has fallen to zero, and the enzyme is all liberated.

The calibrations above indicated that  $p_{\max.} = 0.85 \times 10^{-6}$  mole of hematin Fe per liter in this experiment.

On the left side of Fig. 3 is shown the rate of production of malachite green by the enzyme system under identical conditions. Here again the break in the base-line indicates a push of the syringe plungers. However, the very rapid upward deflection in this case simply represents clearing out malachite green from the previous run. After 0.1 second the flow stops and the production of malachite green begins just as soon as the intermediate compound has formed. The reaction continues at nearly constant velocity as long as the concentration of the intermediate compound is constant. (The slight variation in slope is due to experimental error.) As this falls, so falls the rate of the over-all reaction, and both reach zero at approximately the same time. Calibrations given above indicated that  $4 \times 10^{-6}$  mole of malachite green was formed in this experiment.

This very simple experiment gives qualitative indication that the relationship between the kinetics of the enzyme-substrate compound and the over-all enzyme activity is that predicted by the Briggs and Haldane modifications of the Michaelis theory.

These experiments have been carried out for substrate concentrations ranging from  $5 \times 10^{-7}$  to  $8 \times 10^{-6}$  mole per liter. At the lower concentrations the error in recording was somewhat large, and at those higher than  $6 \times 10^{-6}$  mole per liter the transmission change due to the formation of the quantity of malachite green interfered with measurements of the enzyme kinetics (see "Controls" above). Enzyme concentrations ranged from  $2.5 \times 10^{-7}$  to  $2 \times 10^{-6}$  M hematin Fe. Lack of an adequate supply of enzyme limited the highest concentrations to  $2 \times 10^{-6}$  M hematin Fe.

### Interpretation

*Calculation of  $k_3$* —The "Appendix" gives methods for determining  $k_3$  from the over-all reaction (Equations 9 and 12) and from the enzyme-substrate kinetics (Equations 11, 13, and 16).

The rate of the *over-all reaction* is  $4.3 \times 10^{-6}$  mole of malachite green per second and  $p_{\max.} = 0.85 \times 10^{-6}$  mole per liter. From Equations 9 and 12,  $k_3 = 5.1 \text{ sec.}^{-1}$ .

From the *enzyme-substrate kinetics* there are available the following data for Equation 13.

$$k_1 = 1 \times 10^7 \text{ liter mole}^{-1} \text{ sec.}^{-1}; x_0 = 4 \times 10^{-6} \text{ mole per liter.}$$

$$p_{\max.} = 0.85 \times 10^{-6} \text{ mole per liter and } k_2 = 0.2 \text{ sec.}^{-1}.$$

$\int_0^t p dt$  is evaluated graphically at  $t = 0.24$  second when  $p = p_{\max.}$  and found to be  $0.17 \times 10^{-6}$  mole second; hence  $k_3 = 4.3 \text{ sec.}^{-1}$  for  $k_2 = 0$  and  $4.2 \text{ sec.}^{-1}$  for  $k_2 = 0.2 \text{ sec.}^{-1}$ .

$\int_0^t p dt$  also may be evaluated graphically at  $t = \infty$  when  $p = 0$  and  $x = 0$ . The integral is found to be  $0.84 \times 10^{-6}$  mole second and on substitution in Equation 11,  $k_3 = 4.8 \text{ sec.}^{-1}$ .

According to Equation 16, the value of  $k_3$  is given by  $x_0/(p_{\max} \cdot t_1)$ . As  $t_1 = 0.9$  second,  $k_3$  is calculated to be  $5.2 \text{ sec.}^{-1}$ .

The rate of breakdown of the enzyme-substrate compound in the presence of ascorbic acid is determined from the data of Fig. 6. Using convenient Equation 16, we find in Fig. 8 that the variation of  $k_3$  with ascorbic acid is of such a nature that  $k_3$  divided by the ascorbic acid concentration gives a constant indicative of a second order combination of acceptor and enzyme-substrate compound. The same relationship held for leucomalachite green, and the corresponding quotient is  $3 \times 10^5 \text{ liter mole}^{-1} \text{ sec.}^{-1}$ .

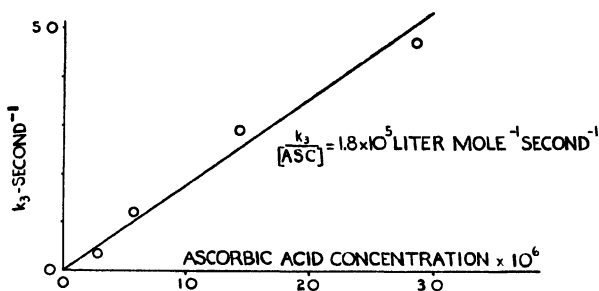


FIG. 8. Variation of  $k_3$  with acceptor concentration.  $k_3$  was obtained by Equation 16 from data of Fig. 6

The constancy of  $k_3$  for a given acceptor concentration is given in Fig. 9 for the data of Fig. 6 on the basis of Equation 16. The experimental check of the equation is satisfactory although the acceptor concentration was somewhat depleted in two reactions with higher substrate concentrations.

A particular curve for  $x_0 = 1.0 \times 10^{-6}$  mole per liter has been examined and  $k_3$  at  $14 \times 10^{-6} \text{ M}$  ascorbic acid is found to be 2.2, 2.0, and  $2.5 \text{ sec.}^{-1}$  from Equations 16, 11, and 13 respectively.

There is then substantial agreement between values of  $k_3$  calculated from three different points of the enzyme-substrate kinetics corresponding to the times  $p = p_{\max}$ ,  $p = p_{\max}/2$ , and  $p = 0$  ( $t = \infty$ ) and between values of  $k_3$  determined from the over-all reaction.

*Calculation of Michaelis Constant*—There are three ways by which we can determine the Michaelis constant and thereby check the validity of the theory.

The first method is to calculate this constant from  $k_2$ ,  $k_3$ , and  $k_1$  which have all been experimentally determined.  $k_3$ , calculated solely from the kinetics of the enzyme-substrate compound above, is found to be  $4.2 \text{ sec.}^{-1}$ .  $k_1$  is found to be  $1 \times 10^7 \text{ liter mole}^{-1} \text{ sec.}^{-1}$  and  $k_2$  a minimum value of  $0.2 \text{ sec.}^{-1}$ . The Michaelis constant is then calculated to be  $0.44 \times 10^{-6}$  from Equation 8.

This value may also be calculated according to Equation 8 from concentrations which obtain during the steady state. The saturation of the enzyme,  $p_{\max}$ , is known from the experiment and the corresponding value of  $x$  may be readily determined. It is important to note that the value of  $x$  is not the initial concentration of substrate as is usually

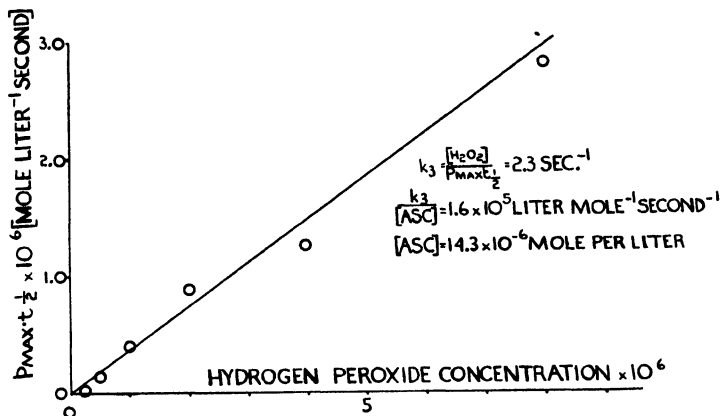


FIG. 9. Experimental test of Equation 16 indicating constancy of  $k_3$  for varying substrate concentration and fixed acceptor concentration. From the data of Fig. 7

the case when this initial concentration is so large compared with the enzyme concentration that the amount of substrate combined with enzyme is relatively small. The value of  $x$  when  $p$  reaches  $p_{\max}$  is calculated from the experimental data in three ways which follow: (a)  $x$  is readily calculated from Equation 11, as we have already determined the necessary quantities.  $p_{\max}$  is equal to  $0.85 \times 10^{-6}$  mole per liter,  $\int_0^{0.24} p dt$  is  $0.17 \times 10^{-6}$  mole second, and  $k_3$  is  $4.2 \text{ sec.}^{-1}$ .  $x$  is  $2.4 \times 10^{-6}$  mole per liter and  $K_m$ , calculated from Equation 8, is  $0.43 \times 10^{-6}$  mole per liter. This method is, of course, not independent of the calculation of  $k_3$  shown previously; nevertheless, all the data used to determine  $K_m$  in this manner are obtained from concentration measurements of the enzyme-substrate compound during the steady state. (b) The amount of sub-

strate which has been consumed by the time  $p$  reaches its maximum value can be determined from Fig. 3 (right) by a second graphical method. The area under the whole curve for the kinetics of the enzyme-substrate compound represents the disappearance of  $4 \times 10^{-6}$  mole of substrate. In fact, this is true for  $\int_0^\infty p dt = 0.84 \times 10^{-6}$  mole second and the mean value of  $k_3 = 4.9 \text{ sec.}^{-1}$ , whence  $k_3 \int_0^\infty p dt = 4.0 \times 10^{-6}$  mole, the initial substrate concentration. The area under the curve from time zero until  $p$  reaches its maximum value is representative of the amount of substrate which has been decomposed during that time and this is  $0.9 \times 10^{-6}$  mole of decomposed substrate. To this we must add the amount of substrate which is combined with the enzyme,  $p_{\max.}$ . From this,  $x$  is readily calculated and the Michaelis constant is found to be  $0.40 \times 10^{-6}$ . This method is completely independent of a determination of  $k_3$ , as this quantity appears in both numerator and denominator. (c) If we assume that for each molecule of malachite green formed 1 molecule of substrate has been decomposed, we have directly the amount of substrate that disappeared enzymatically. At 0.24 second this is  $0.9 \times 10^{-6}$  mole. When  $p_{\max.}$  is added to this, the Michaelis constant is calculated to be  $0.40 \times 10^{-6}$ , which agrees very closely with the other values determined independently.

The classical determination of the Michaelis constant by Mann (12) gives  $5 \times 10^{-6}$  mole per liter at pH 4.0 and an acceptor concentration of 0.007 per cent. This constant varied linearly with acceptor concentration over this range. These data also indicated a linear relationship. Hence Mann's value of  $K_m$  was reduced to our acceptor concentration by dividing by the concentration differential, 10. This gives  $0.5 \times 10^{-6}$ , which agrees fairly well with the above independently determined values in view of the widely different enzyme and substrate concentrations.

*Correlation with Complete Solutions of Michaelis Theory*—While previous data suggest the validity of the Michaelis theory, a much more convincing proof is furnished by the data on the superposition of the differential analyzer (see "Appendix") and direct experimental curves.

The solid curves in Fig. 10 show the kinetics of the enzyme-substrate compound (right) and the over-all reaction (left) for the following values of reaction velocity constants and concentrations:  $e = 1 \times 10^{-8}$  mole per liter,  $x_0 = 4 \times 10^{-6}$  mole per liter,  $k_1 = 0.9 \times 10^7$  liter mole $^{-1}$  sec. $^{-1}$ ,  $k_2 = 0$  sec. $^{-1}$ ,  $k_3 = 4.5$  sec. $^{-1}$ . The experimental curves of Fig. 3 ( $e = 1 \times 10^{-8}$  mole of hematin Fe per liter,  $x_0 = 4 \times 10^{-6}$  mole per liter, leucomalachite green =  $15 \times 10^{-6}$  mole per liter, pH = 4.0) are plotted as circles to the proper scale in Fig. 10. The independently determined

values of reaction velocity constants are  $k_1 = 1.2 \times 10^7$  liter mole<sup>-1</sup> sec.<sup>-1</sup>,  $k_2 \cong 0.2$  sec.<sup>-1</sup>,  $k_3 = 4.9$  sec.<sup>-1</sup> (mean). Remarkably good agreement is obtained in view of the possible error in all experimental quantities required to determine the mathematical solution.

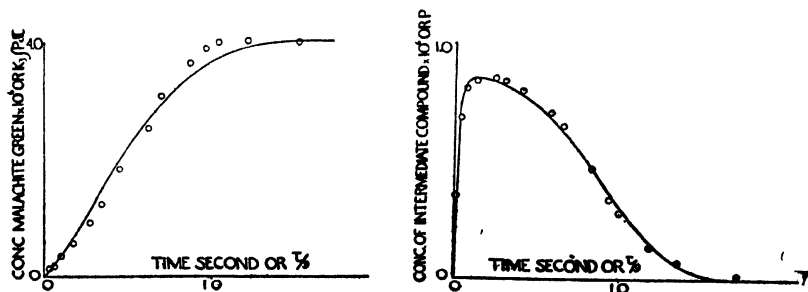


FIG. 10. A comparison of experimental enzyme-substrate and "over-all" kinetics (circles) with a mathematical solution of the Michaelis theory for experimentally determined reaction velocity constants and concentrations (solid lines).

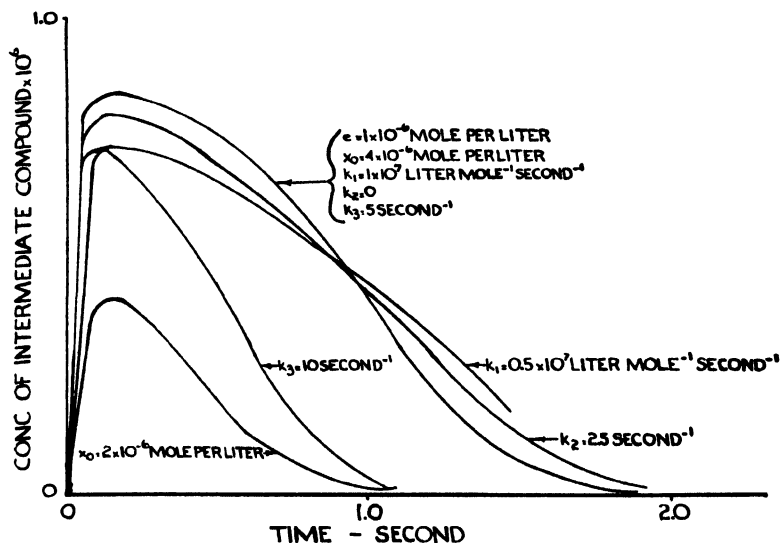


FIG. 11. Effect of variation of reaction velocity constants and concentrations on the shape of the mathematical solutions of the Michaelis theory.

The overshoot in the experimental points (Fig. 10, right) is possibly due to inadequate speed of response in the photocell amplifier. The scatter of points (Fig. 10, left) is thought to represent an instrumental rather than intrinsic irregularity.



Calculations show that the mathematical solutions are quite sensitive to changes in the experimental values.  $x_0$  and  $k_3$  cause large changes in  $t_1$ , while  $k_1$  and  $k_2$  affect  $p_{\max}$  and the shape of the curve as shown in Fig. 11. The effect of enzyme and leucomalachite green concentration is not given by the mathematical solutions but would be large.

#### DISCUSSION

The extreme rapidity of the union of enzyme and substrate is indicated by the second order rate constant of  $1 \times 10^7$  liter mole<sup>-1</sup> sec.<sup>-1</sup>. It is very interesting to note the similarity of this rate to the measured value for oxygen and muscle hemoglobin,  $1.9 \times 10^7$  liter mole<sup>-1</sup> sec.<sup>-1</sup> (Millikan (14)). Haldane's (9) calculated minimum rate for the union of catalase and hydrogen peroxide of  $0.76 \times 10^7$  liter mole<sup>-1</sup> sec.<sup>-1</sup> is quite similar also. If it is assumed that the reversible breakdown of the enzyme-substrate compound is also slow in the case of catalase and hydrogen peroxide, the similarity between the catalase and peroxidase values is more striking. Such concordance in the rates of union of small molecules and proteins would lead one to question whether or not these reaction velocities were limited by the number of collisions. While experiments made at 0° indicate but little change in the rate of formation of the enzyme-substrate compound, these results are preliminary and should not be used to substantiate the conclusion that the joining of enzyme and substrate is a collision-limited process.

The equilibrium of enzyme and substrate was directly studied as the irreversible breakdown of the intermediate compound ( $k_3$ ) was quite small in the absence of acceptor. The equilibrium constant was found to have a minimum value of  $2 \times 10^{-8}$ . This indicates an extremely tight complex of enzyme and substrate, and this value is of the same order as that for CO hemoglobin, although the individual rates differ considerably. Cytochrome *c* peroxidase-hydrogen peroxide evidently dissociates more readily (1).

The studies on the enzymatic function of the enzyme-substrate compound were also carried out with ascorbic acid and leucomalachite green as acceptor, the latter over a rather narrow range, as the absorption of dye interfered with the measurement of the enzyme-substrate compound. The Michaelis theory has been checked by various determinations of the Michaelis constant. The first method is from *kinetic* data; namely, the rate of formation of the enzyme-substrate compound, the rate of reversible breakdown, and the rate of its irreversible breakdown into free enzyme and altered substrate. The Michaelis constant was determined from the sum of the last two divided by the first. This was also determined from *concentration measurements* at the steady state, when the concen-

tration of the intermediate compound passed through its maximum value. From this maximum value and the corresponding substrate concentration we can again directly calculate the Michaelis constant. The third method is the classical one wherein the rate of the over-all reaction is measured, and the concentration of substrate giving one-half maximal activity is determined. To these three methods a fourth one has been added to take advantage of the fact that the data are complete kinetic curves of the enzyme-substrate compound, and therefore, with complete solutions of the Michaelis equation we may compare, point by point, theory and experiment.

In all cases in the range of experimental concentrations the kinetics of the intermediate compound were related to the kinetics of the over-all reaction in a manner explained by the Michaelis theory, substantiating the conclusion that the mechanism of a second order combination of enzyme and substrate followed by a first order decomposition is essentially correct for peroxidase action at this particular acceptor concentration.

The rate of breakdown of the intermediate compound of peroxidase and hydrogen peroxide is very small compared to that of catalase  $k_3 = 3 \times 10^4 \text{ sec.}^{-1}$ . The value for catalase assumes that the Michaelis theory holds and that a chain mechanism is not responsible for the enzyme action. The peroxidase kinetics indicate that a chain mechanism plays no prominent part, if any, as the induction period in the production of dye is no longer than is required by the formation of the enzyme-substrate compound and there is also no further production of dye after the enzyme-substrate compound has disappeared. It is possible that the difference between these two enzymes lies mainly in the slower breakdown of the peroxidase intermediate compound.

It is of considerable interest to know whether there is a bimolecular combination of the enzyme-substrate compound and the acceptor. No spectroscopic evidence of such compound formation from 360 to 600  $m\mu$  was found. However, kinetic evidence for such a combination is given by the variation of the enzyme activity with acceptor concentration. The rate of production of malachite green and the effect of ascorbic acid on the kinetics of the enzyme-substrate compound strongly suggest a bimolecular combination with acceptor in accordance with Mann (12).

The mechanism by which the acceptor is oxidized is still obscure. As this may take place through single electron changes involving the formation of a free radical of the triphenylmethyl type in the case of malachite green, studies were made<sup>3</sup> to find spectroscopic evidence for such intermediates. While no data were obtained in the visible spectrum, the question is still open.

<sup>3</sup> Dr. Fred Karush collaborated in this study.

## SUMMARY

Under the narrow range of experimental conditions, and at a temperature of approximately 25°, the following data were obtained.

1. The equilibrium constant of peroxidase and hydrogen peroxide has a minimum value of  $2 \times 10^{-8}$ .

2. The velocity constant for the formation of peroxidase- $\text{H}_2\text{O}_2$  Complex I is  $1.2 \times 10^7$  liter mole $^{-1}$  sec. $^{-1}$ ,  $\pm 0.4 \times 10^7$ .

3. The velocity constant for the reversible breakdown of peroxidase- $\text{H}_2\text{O}_2$  Complex I is a negligible factor in the enzyme-substrate kinetics and is calculated to be less than 0.2 sec. $^{-1}$ .

4. The velocity constant,  $k_3$ , for the enzymatic breakdown of peroxidase- $\text{H}_2\text{O}_2$  Complex I varies from nearly zero to higher than 5 sec. $^{-1}$ , depending upon the acceptor and its concentration. The quotient of  $k_3$  and the leucomalachite green concentration is  $3.0 \times 10^5$  liter mole $^{-1}$  sec. $^{-1}$ . For ascorbic acid this has a value of  $1.8 \times 10^5$  liter mole $^{-1}$  sec. $^{-1}$ .

5. For a particular acceptor concentration,  $k_3$  is determined solely from the enzyme-substrate kinetics and is found to be 4.2 sec. $^{-1}$ .

6. For the same conditions,  $k_3$  is determined from a simple relationship derived from mathematical solutions of the Michaelis theory and is found to be 5.2 sec. $^{-1}$ .

7. For the same conditions,  $k_3$  is determined from the over-all enzyme action and is found to be 5.1 sec. $^{-1}$ .

8. The Michaelis constant determined from kinetic data alone is found to be  $0.44 \times 10^{-6}$ .

9. The Michaelis constant determined from steady state measurements is found to be  $0.41 \times 10^{-6}$ .

10. The Michaelis constant determined from measurement of the over-all enzyme reaction is found to be  $0.50 \times 10^{-6}$ .

11. The kinetics of the enzyme-substrate compound closely agree with mathematical solutions of an extension of the Michaelis theory obtained for experimental values of concentrations and reaction velocity constants.

12. The adequacy of the criteria by which experiment and theory were correlated has been examined critically and the mathematical solutions have been found to be sensitive to variations in the experimental conditions.

13. The critical features of the enzyme-substrate kinetics are  $p_{\text{max}}$  and curve shape, rather than  $t_1$ .  $t_1$  serves as a simple measure of  $dx/dt$ .

14. A second order combination of enzyme and substrate to form the enzyme-substrate compound, followed by a first order breakdown of the compound, describes the activity of peroxidase for a particular acceptor concentration.

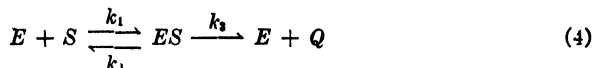
15. The kinetic data indicate a bimolecular combination of acceptor and enzyme-substrate compound.

It is a very great pleasure to acknowledge the aid of Dr. F. J. W. Roughton, Dr. F. A. Cajori, Dr. G. A. Millikan, and Dr. J. G. Brainerd, and the keen interest of Dr. D. W. Bronk in this research. The aid of the American Philosophical Society is gratefully acknowledged. It is also a source of regret that the problem could not be concluded where it was initiated.

### Appendix

#### *Extension of Michaelis Theory*

These reactions are represented by Briggs and Haldane as the bimolecular combination of the enzyme,  $E$ , and substrate,  $S$ , to form an intermediate compound,  $ES$ , followed by a monomolecular decomposition into free enzyme and activated or altered substrate,  $Q$ , representative of the products of the "over-all" enzyme action.



If  $e$  is the total molar enzyme concentration,  $x$  the molar substrate concentration,  $p$  the molar concentration of  $ES$ ,  $k_1$  the second order rate constant, and  $k_2$  and  $k_3$  the first order rate constants, then

$$\frac{dp}{dt} = k_1 x(e - p) - (k_2 + k_3)p \quad (5)$$

$$\frac{dx}{dt} = -k_1 x(e - p) + k_2 p \quad (6)$$

These two equations represent the rate of formation of the intermediate compound and the rate of disappearance of the substrate.

The solution of these equations has been already obtained by Briggs and Haldane for the special conditions of the steady state, when

$$p = p_{\max}, \quad \frac{dp}{dt} = 0, \quad \text{and} \quad \frac{p_{\max}}{e} = \frac{x}{x - Km} \quad (7)$$

where

$$Km = \frac{k_2 + k_3}{k_1} = x \frac{(e - p_{\max})}{p_{\max}} \quad (8)$$

A further solution valid during the steady state is obtained by adding Equations 5 and 6,

$$\frac{dx}{dt} = -k_3 p_{\max} \quad (9)$$

where  $dx/dt$  is the rate of disappearance of substrate. This equation is useful for determining  $k_3$ .

In addition to these solutions for the steady state the general solution of these differential equations can be indicated thus:

$$\frac{d(p+x)}{dt} = -k_3 p \quad (10)$$

and is obtained by adding Equations 5 and 6. As  $p = 0$  and  $x = x_0$  when  $t = 0$ , we have, on integrating,

$$x = x_0 - p - k_3 \int_0^t p dt \quad (11)$$

When  $dp/dt = 0$ ,

$$\frac{dx}{dt} = -k_3 \frac{d \int_0^t p dt}{dt} \quad (12)$$

The right-hand member may represent the rate of appearance of oxidized substrate such as malachite green. We may then use Equation 9 to calculate  $k_3$ .

Also when  $dp/dt = 0$ ,  $p = p_{\max}$  and at this time Equation 7 is valid. On solving Equation 7 for  $x$  and substituting in Equation 12, we have, after simplification,

$$k_3 = \frac{k_1(x_0 - p_{\max.})(e - p_{\max.})}{p_{\max} + k_1(e - p_{\max.}) \int_0^t p dt} - \frac{k_2 p_{\max}}{p_{\max} + k_1(e - p_{\max.}) \int_0^t p dt} \quad (13)$$

This equation is useful to calculate  $k_3$  when the curve of  $p$  against  $t$  is known as in the case of Fig. 3.

As a check on the mathematics, let us substitute in Equation 13 the condition that  $p_{\max.}$  is nearly equal to  $e$ ,  $t$  is small, and  $x$  is nearly equal to  $x_0$ . It will be seen that Equation 7 is obtained as would be expected.

*Complete Solutions for Michaelis Theory*<sup>4</sup>—Under the experimental conditions it was found that the steady state existed only for a fraction of a second. In order to determine whether the transient portions of the curves satisfied the Michaelis theory, solutions of differential Equations 5 and 6 were required. For satisfactory solutions from the differential analyzer, the following substitutions were necessary. Let

$$\begin{aligned} K_2 &= 10^6 \frac{k_2}{k_1}, & X &= 10^6 x, & \tau &= k_1 e t \\ K_3 &= 10^6 \frac{k_3}{k_1}, & P &= \frac{p}{e}, & \Theta &= k_1 t 10^{-6} \end{aligned}$$

<sup>4</sup> These solutions were obtained with the aid of Dr. J. G. Brainerd, Moore School, University of Pennsylvania.

$$\therefore \frac{dP}{d\Theta} = (1 - P)X - (K_2 + K_3)P \quad (14)$$

$$\frac{dX}{d\tau} = -(1 - P)X + K_2P \quad (15)$$

When  $e$  is equal to  $1 \times 10^{-6}$  mole per liter, the experimental value, then  $\tau$  is equal to  $\Theta$  and a series of solutions of these equations may be obtained for this particular value of the enzyme concentration. The solutions have been carried out in this manner, and it should be noted that they are valid only for this enzyme concentration.

The mathematical solutions are given in Fig. 12, and solutions have been obtained for  $K_2 = 0$ ;  $K_3 = 0$  to 2; and  $X_0 = 1$  to 8. Solutions for  $K_2 = 0$  to 8 and  $K_3 = 0$  to 8 were also made.

The upper portions of Fig. 12 show the disappearance of substrate,  $X$ , and production of "over-all" products,  $Q$ . The substrate concentration starts at its initial value,  $X = X_0$ , and falls to zero. The "over-all" production, represented by  $K_3 \int_0^\tau P d\tau$ , begins at zero and continues until the substrate is exhausted.

The lower left portion of Fig. 12 gives the ordinary solutions of the bimolecular reaction of enzyme and substrate, when  $K_2$  and  $K_3$  are zero, for four values of the initial substrate concentration,  $X_0$ . 1 unit of the ordinate corresponds to  $p = e$ ; i.e., complete conversion of the enzyme into the intermediate compound. The abscissae, plotted in units of  $\tau$ , are converted into time units by the appropriate values of  $k_1$ . Directly above this is the disappearance of substrate due to the bimolecular reaction.

In the remaining lower portions of Fig. 12,  $K_2$ , the velocity constant of the reversible reaction is zero and  $K_3$  has a finite value. The concentration of the intermediate compound increases, passes through a maximum, and then falls to zero. The maximum concentration and area under the curve increase with the substrate concentration. Directly above are the corresponding curves for the over-all reaction. The initial rush in the kinetics of the disappearance of substrate and the induction period in the formation of "over-all" products are significant features.

The abscissae in all cases are represented in units of  $\tau$  and therefore a wide range of values of  $k_1$  can be used. The range of values of  $K_2$ ,  $K_3$ , and  $X_0$  is that corresponding to the values for which solutions have been obtained. However, to determine  $k_2$  or  $k_3$ ,  $K_2$  or  $K_3$  is multiplied by  $k_1$ .

For larger values of  $k_1$  and  $X_0$ , and smaller values of  $K_3$ ,  $dp/dt$  will become quite small for a considerable time and solutions corresponding to  $dp/dt = 0$  can easily be obtained, as shown by Briggs and Haldane (2).

The families of curves obtained from the differential analyzer are obviously applicable to any reversible bimolecular combination and a con-

secutive monomolecular breakdown of the intermediate compound. It should be noted that these mathematical solutions do not completely describe peroxidase action, as they do not include the acceptor process. Hence they are valid only for fixed acceptor concentrations giving constant  $k_3$ .

A useful feature of these mathematical solutions is that they reveal arbitrary relationships between members of the families of curves. As an example it is found from measurements of the mathematical solutions

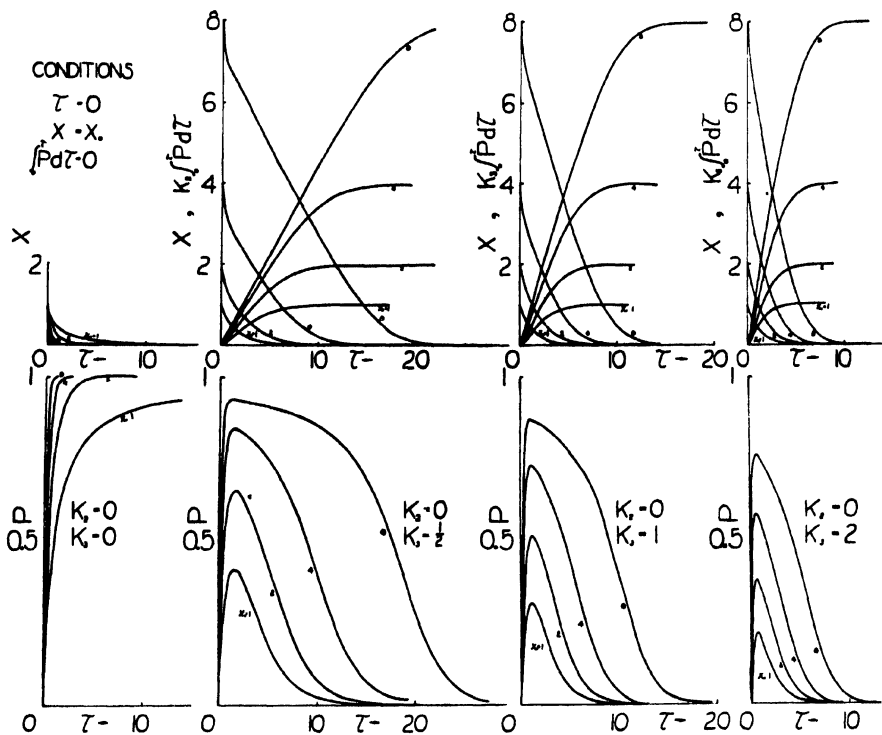


FIG. 12. Differential analyzer solutions of the Michaelis theory for the over-all reaction (upper group) and the kinetics of the enzyme-substrate compound (lower group).

for the kinetics of the enzyme-substrate compound that  $k_3$  can be determined directly by this formula if the acceptor concentration is constant.

$$k_3 = \frac{x_0}{p_{\max.} t_1} \quad (16)$$

$p_{\max.}$  is the maximum value of  $p$ .  $t_1$  is the time required for  $p$  to fall from  $p_{\max.}$  to  $p_{\max.}/2$ . This relationship has been tested for experimental curves and found very satisfactory, as shown in Fig. 9.

If Equation 16 is combined with Equation 9, we find

$$t_1 = - \frac{x_0}{dz/dt} \quad (17)$$

This indicates that there are a variety of curves of the same half width that would satisfy our experimental data. The shape and  $p_{\max}$  would be quite different.

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## PURIFICATION OF INTERMEDIN

### I. RECOVERY AND PURIFICATION FROM WASTE FRACTIONS OF SHEEP<sup>5</sup> PITUITARY GLANDS

By A. A. ABRAMOWITZ, D. N. PAPANDREA, AND F. L. HISAW

(From the Biological Laboratories, Harvard University, Cambridge)

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Intermedin is the term introduced by Zondek and Krohn (1) to describe a hormone arising from the intermediate lobe of the pituitary gland and possessing the property of expanding the erythrophores of the minnow, *Phoxinus*. The relation of intermedin to another well established pituitary chromatophorotropic hormone, the melanophore hormone, was imperfectly understood by the above authors, but subsequent workers (2, 3) claimed to have effected a separation of the two principles. Recent work (4), however, has shown that there is indeed no valid basis for the existence of two such principles, and, in consequence, the prevailing opinion now favors the belief that the pituitary secretes one chromatophorotropic hormone which is capable of provoking pigment migration within various chromatophores (erythrophores, xanthophores, melanophores, etc.). For many reasons, the term intermedin has been retained by many workers to denote the chromatophorotropic principle of the pituitary. For additional details concerning the physiological aspects of this hormone, the reader is referred to several recent comprehensive articles (5-7).

The chemistry of intermedin has long been neglected, and there exists only a fragmentary and incomplete knowledge of its chemical properties. Intermedin is water-soluble, heat-stable, and dialyzable, three properties upon which all investigators are in agreement. Its solubility in alcohols is affirmed by some, denied by others. With the exception of the lower alcohols, however, intermedin apparently is insoluble in all common organic solvents. It is remarkably stable to strong alkali, even when exposed at 100° (for some time, at least), and many authors have reported that such treatment actually potentiates the physiological effect of the hormone. Only fragmentary data exist with regard to its behavior towards mineral acids. Some authors considered intermedin to be a poorly adsorbable substance; others, on the other hand, have considered it to be strongly adsorbable. It has been known for about 20 years that the physiological activity of intermedin is lost after tryptic digestion of an intermedin preparation. This fact, in conjunction with the ability of intermedin to pass through natural and artificial membranes, has led to the opinion that intermedin is a polypeptide. However, in the absence of any data establishing the enzy-

matic nature of the inactivation of intermedin by trypsin, the proof that the hormone has a peptide-like structure is not wholly complete.

Several attempts have been made to purify and isolate intermedin. Zondek and Krohn speak of its isolation, but it is doubtful whether they had effected even a substantial purification of the material. Stehle (8) and Böttger (9) have published methods for securing a preparation of the hormone 25- and 50-fold purified respectively. All three methods involve preliminary extraction of the hormone by boiling pituitary glands or dried pituitary powders in dilute acetic acid, dissolution of the extract in alcohol, and precipitation of the active material from alcoholic solution by the addition of some organic solvent such as ether, acetone, or ethyl acetate.

The purpose of this paper is to describe several methods by which preparations of intermedin several hundred times purer than the original gland tissue have been obtained. In addition, many new chemical properties of the hormone are described. Owing to the cost of pituitary tissue, most of this investigation was carried out on intermedin salvaged from waste fractions of a process of extracting pituitary tissue for the gonadotropic hormones (10). Only small amounts of intermedin were salvaged, but by accumulating the material over a period of time sufficient hormone was obtained to make the study worth while. Another difficulty encountered was that the methods for extracting and purifying the gonadotropic hormones were being constantly altered and improved (11). The sources for the intermedin therefore were different from time to time, with the result that certain changes in the recovery procedure had to be improvised.

### Methods

The chemical procedures employed will be dealt with below. The method of assay will be described briefly. Frogs (*Rana pipiens*) are placed in a large box (30 × 30 × 20 inches), the sides and floor of which were covered with white oilcloth. Two 150 watt electric lamps were suspended over the box so that the animals were illuminated continuously. 3 or more days in such surroundings sufficed to induce a marked pallor (greenish yellow color) of the frogs, and only those possessing a more or less uniform color of the skin were selected for assays. The solution to be tested was injected in the dorsal lymph sac (0.5 cc. total volume) of two frogs, and  $\frac{1}{2}$  hour later the animals were observed to determine whether a minimal positive response (darkening of the entire skin) had occurred. If positive, progressive dilutions of the solution were injected until a point was reached at which the frogs no longer responded. The dilution just preceding this point was accepted as the end-point, and the total number of frog units was calculated from the dilution. The dilutions were made geometrically ( $X$ ,  $X/2$ ,  $X/4$ ,  $X/8$ , etc.), so that the accuracy of the assay could be in theory no greater

than  $\pm 100$  per cent. However, this simplified routine method need not be more accurate in the early stages of purification, since the methods to be described yielded almost total recoveries of the hormone. However, if after a fractionation the original activity was found to be seriously divided, larger numbers of frogs (six to ten) were injected with several dilutions bridging the 100 per cent gap between a dose of  $X$  (giving a positive response) and  $X/2$  (giving no response). Thus more critical assays were obtained.

#### EXPERIMENTAL

*Standard Tissue Powder*—Commercial sheep pituitary powder assays at  $100 \times 10^6$  frog units per kilo, or 0.1 frog unit per microgram of solids. Fresh, frozen, sheep pituitary glands assay at  $50 \times 10^6$  frog units per kilo of wet weight. 80 per cent, however, is usually the water content of these glands; hence the activity of the fresh tissue in terms of solids is  $200 \times 10^6$  frog units per kilo, or 0.2 frog unit per microgram of solids. Thus fresh tissue is about twice as potent with respect to intermedin as commercial pituitary powder. The loss of half the hormone content is probably due to the fact that the glands are dehydrated with acetone and passed through bolting cloths when prepared as commercial powders. The above figures are averages in round numbers of several batches of tissue, whether powdered or fresh. Actually, however, the intermedin content varies from batch to batch.

To describe the extent of purification, the term specific activity will be used. Specific activity will be defined as the number of frog units of intermedin per microgram of solids of any preparation. Small aliquots of any preparation were dried at  $110^\circ$  until a constant weight was attained. The specific activity of fresh tissue is therefore 0.2, that of powder 0.1.

*Initial Extraction*—1 kilo of fresh sheep pituitary glands, frozen with dry ice, is ground in an electric mill to a fine powder. 2 liters of 2 per cent pyridine are added with stirring to emulsify the tissue powder. After the mixture has stood overnight at room temperature, 10 per cent acetic acid is added until the pH of the emulsion is 5.0, and the emulsion centrifuged. The pinkish supernatant fluid is decanted and stored while the residue is reextracted with 1 liter of 2 per cent pyridine in the same fashion. After the pH is adjusted to 5.0 with acetic acid, and the material centrifuged, the supernatant fluid is combined with that of the first extraction. This method had been in use in this laboratory (10) for some time. It yields about 4 liters of an extract that contains the gonadotropic, thyrotropic, and other anterior lobe hormones, and from 75 to 90 per cent of the total intermedin content. The residue (insoluble material at pH 5.0) of the two extracts had always been discarded, but it was found that it contained from one-fourth to one-tenth of the total intermedin content (the specific activity

varied from 0.03 to 0.07) and that the hormone could easily be salvaged. The recovery of intermedin from both the residue and from waste fractions of the extract will be described.

*Recovery of Intermedin from Residue*—The residue described above is emulsified in 2 liters of water, the pH of the emulsion adjusted to 5.0 if necessary with 10 per cent sulfuric acid, and the mixture boiled for 15 minutes. On boiling, the emulsion breaks into a yellowish clear solution and a heavy gray precipitate. The solution is filtered while hot through a coarse filter. The residue is collected and boiled again in a liter of water (acidulated to pH 5.0). This extract after filtration is combined with the first, yielding about 3 liters of a clear yellowish solution. The solution is then poured into large, round evaporating dishes and is evaporated under a hood by means of a warm current of air (40°). As the solution is being evaporated, a yellowish disk of gelatin forms on the surface of the solution. This disk hardens as evaporation proceeds and when dry is removed and placed in a beaker containing 2 liters of cold distilled water (acidulated with  $\text{H}_2\text{SO}_4$  to pH 4.7) and kept at 5° for several days. As subsequent disks form, they are similarly removed until all of the yellowish solution has been evaporated.

The gelatinous disks soften and become whitish with continuous leechings with water. When no more yellow color is extracted, the gelatin is discarded, and the combined cold extracts are evaporated under the hood. This extract contains only minute amounts of gelatin and consequently can be evaporated to uniform dryness. The material when evaporated is a dark brown, hygroscopic substance of a much more favorable appearance than the preceding gelatinous matrix. The hygroscopic material is taken up in 500 cc. of distilled water, centrifuged clear of insoluble material, and stored at 2°. This solution (A) is slightly acid (pH 5.5 to 6.0), slightly turbid, and mahogany-colored, containing 30 to 50 gm. of solids, and possessing a specific activity of 1.1. With a waste fraction containing only 10 to 25 per cent of the total intermedin as a source, the two steps outlined yielded a solution with a specific activity 5 times greater than the pituitary tissue.

About half of the inert material present in Solution A can be removed by either of two methods. Solid recrystallized  $\text{Ba}(\text{OH})_2$  is added slowly to Solution A until the pH is 8.0 to 8.5. A heavy white precipitate of barium salts is centrifuged off. The supernatant fluid (clear, and lemon-yellow in color) is freed of excess barium by means of  $\text{H}_2\text{SO}_4$  and centrifuged free of sulfates. Intermedin remains quantitatively in solution throughout the barium fractionation and removal of excess barium. The specific activity of this solution (B) varies from 2.0 to 2.2.

The second method of removal of almost half of the inert material in

Solution A consists of adding basic lead acetate to the solution until no further precipitation occurred. The exact amount added varies from batch to batch and has to be determined by repeated samplings. When precipitation is complete, the lead salts are removed by centrifugation, and excess lead removed as the sulfate. This yields a clear, greenish yellow solution ( $B_1$ ) with a specific activity of 1.6 to 1.8. This method entails a loss of some intermedin which is probably adsorbed to the discarded lead precipitates. The extent of the loss depends to some degree on the pH of the solution, which should not rise above pH 7.2 when lead acetate is added. More inert material can be removed by fractionating first with barium and then with lead, then with either alone, but the variability of the lead fractionation and the loss of hormone it entails does not make it worth while as a routine procedure. In the most favorable cases, the specific activity of the clear greenish solution ( $B_2$ ) after the double fractionation varied from 2.8 to 3.2.

Solution B,  $B_1$ , or  $B_2$  is neutralized with 5 N NaOH to pH 7.0. 2,4-Sodium phosphotungstate is added with stirring to the hormone solution, and then 1 N  $H_2SO_4$  is added drop by drop until the pH is 3.0. The phosphotungstates are centrifuged off, suspended in distilled water (200 cc.), the pH of the suspension brought to 3.3 to 3.5, and the suspension filtered with suction. The phosphotungstates were decomposed with cold  $Ba(OH)_2$  in the usual manner, excess barium removed as the sulfate, and the solution adjusted to pH 7.0 with NaOH. The hormone was quantitatively recovered throughout the fractionation and concentrated 5-fold, the volume now being 100 cc. The specific activity of this solution (C) was 10 (50 times greater than pituitary tissue and 125 to 350 times greater than the starting source).

When the residues of 10 kilos of fresh pituitary glands received over a period of 5 months had been worked up to this state (Solution C), the initial extraction procedure was changed (11) in that ammonia instead of pyridine was used as extractive. Moreover, the extraction was rendered more thorough by alternate freezing and thawing of the tissue prior to grinding. The residues of the ammonia extract proved to be almost inactive with respect to intermedin content. Since no further source similar to that described could be depended upon, the purified intermedin (Solution C) was used primarily with the view of exploring possible further methods for purification.

*Adsorption Studies*—In view of the successes of adsorption methods in the purification of several organic bases of the vitamin B complex, it seemed worth while to study the adsorption of intermedin. The results of these experiments were unimpressive as far as a gain in specific activity was concerned, but they are of some interest in relation to the previous conflicting reports on the adsorptive properties of the hormone. It was found that

intermedin can be quantitatively removed from acid solutions by small amounts of norit A or fullers' earth. It is only partly removed by synthetic zeolite, and not at all removed by kaolin. The idea of employing either fullers' earth or zeolite as a method of purification seemed at first optimistic, since the latter removed the intermedin with only one-fifth to one-tenth of the solids of the solution. Nevertheless, the hormone could not be satisfactorily eluted although many eluents such as dilute ammonia, acid quinine sulfate, barium hydroxide, and pyridine-water-methanol-acetone mixtures of various proportions were tried. In the best of cases, not more than half the total intermedin could be eluted, and the same difficulty applied to the carbon adsorbate. The intermedin adsorbed on zeolite could be eluted more readily with  $\text{Ba}(\text{OH})_2$  (but not with neutral salts), but the initial loss (intermedin not adsorbing) and the loss entailed during elution deprived the method of any gain in purification. Intermedin is not adsorbed by benzoic acid. These results show that under proper conditions with the proper adsorbing agent intermedin is strongly adsorbed.

*Dialysis*—Intermedin passes readily but not quantitatively through cellophane membranes (Visking Corporation). After several days dialysis against distilled water, the dialysate, though containing less than one-half of the total solids, had no appreciable gain in specific activity, owing to the retention of considerable activity within the membrane.

10 per cent silver nitrate was added in slight excess to Solution C previously brought to pH 5.0 with 10 per cent  $\text{HNO}_3$ . A considerable precipitate of both inorganic and purine silver compounds occurred with no loss of activity. Intermedin remains quantitatively in solution if the pH does not rise above 4.0 to 4.5. Addition of  $\text{Ba}(\text{OH})_2$  to pH 8.5 precipitated most of the intermedin, which can be recovered in its free form by decomposing the silver precipitate with  $\text{HCl}$ . The acid decomposition must be carefully and rapidly carried out, since excess  $\text{HCl}$  seemed to cause loss of activity. A purification of 100 per cent can be effected over Solution C, the specific activity now being 20 (Solution D).

*Fractionation with Alcohol*—Addition of ethyl alcohol to Solution D to 60 per cent by volume resulted in a white bulky precipitate with only a negligible specific activity (10 per cent of the total intermedin concentration). The alcoholic solution was made alkaline to tropeolin O with 5 N  $\text{NaOH}$ , and more alcohol was added to 80 per cent by volume. The solution was thoroughly shaken and centrifuged free of insoluble material which was subsequently discarded. The cautious addition of N  $\text{HCl}$  or acetic acid to the alkaline alcoholic solution resulted in a fine precipitate containing 80 per cent of the intermedin of the solution, and with a specific activity of 40. As compared with pituitary tissue, intermedin has now been purified 200 times, and as compared with the starting material, 700 to 1300 times. The

addition of acid must be carried out carefully, since the isoelectric region is slightly above neutrality, and excess acid tends to redissolve the precipitate. The discovery of this behavior of the hormone suggested a possible method of crystallization, but when purer material, to be described later, is employed, it is doubtful whether this represents the behavior of the hormone itself. More likely, isoelectric precipitation of intermedin from alcoholic solution represents the behavior of some other constituent to which intermedin is either adsorbed or combined. As an indication of the correctness of this view, a small portion of the isoelectric precipitate was dissolved in water and an equal volume of 10 per cent trichloroacetic acid added. An inactive precipitate immediately formed, which was removed by filtration. The material in the acid filtrate after neutralization was no longer precipitated from alcoholic NaOH by either acetic or hydrochloric acid.

*Recovery from Pyridine Extracts of Sheep Pituitary Glands. Precipitation by Means of Alcohol*—To the 3 liters of the combined pyridine-acetic acid extracts sufficient alcohol was added to make a 75 per cent alcoholic solution by volume, and the solution filtered. The precipitate was washed several times with liter portions of 75 per cent ethanol, the washings being added to the first alcoholic extract. This alcoholic solution contains half of the intermedin present in the pyridine extract, or from 35 to 45 per cent of the total intermedin content of the pituitary tissue. The precipitate contains the gonadotropic, thyrotropic, and other hormones of higher molecular weight.

The alcoholic solution was evaporated to dryness under the hood, yielding a hygroscopic dark brown material. This was dissolved in a liter of water, boiled for 15 minutes, and filtered. The brown filtrate had a specific activity of 1.6; the total solids from 1 kilo of tissue averaged 20 gm. Fractionation of the filtrate with barium, phosphotungstic acid, and silver nitrate, in the manner already described, yielded a clear solution of intermedin with an activity of 60 to 80, or a purification of 300 to 400 times that of pituitary tissue.

*Recovery of Intermedin by Dialysis*—In place of alcoholic precipitation, the pyridine extract can be dialyzed to collect appreciable intermedin. However, this method is slow and somewhat inconvenient. The specific activity of the dialysate naturally varies with the extent of the dialysis, but compares favorably with that of the alcoholic extract.

#### SUMMARY

Several methods are described for the recovery and purification of intermedin from certain fractions ordinarily discarded during the preparation of gonadotropic and other hormones of the pituitary. The gland residue (after initial extraction for the gonadotropic hormones) is boiled, gelatin



removed by leeching with cold distilled water, and further inert material removed as lead or barium salts. Intermedin is precipitated from solution by phosphotungstic acid, fractionated with silver, and finally with alcohol. The resulting product represents a purification of 200-fold over that of pituitary tissue, or from 700 to 1300 times that of the starting material.

Similar treatment of the alcoholic filtrate of the initial pyridine extract can be worked up to yield a 300 to 400 increase in purity over that of the original tissue.

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# COLORIMETRIC DETERMINATION OF THE ESTROGENS: A METHOD FOR THE DETERMINATION OF TOTAL ESTRONE AND ESTRADIOL FROM TISSUE SOURCES\*

BY CLARA M. SZEGO AND LEO T. SAMUELS

*(From the Divisions of General Physiology and Physiological Chemistry, Department of Physiology, University of Minnesota Medical School, Minneapolis)*

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Specific colorimetric methods for the individual estrogens which could be applied to biological systems would be valuable tools in the study of steroid metabolism.

The guaiacolsulfonic acid method, as published from this laboratory (1), serves to distinguish estrone in the presence of estriol. The quantities of estradiol presumably present in the biological extracts to which this method was expected to be applied did not interfere appreciably with the estimation of estrone. Larger quantities, of the order of 10 to 50  $\gamma$ , however, gave significant amounts of yellow to pink color. The variability of the estradiol color by this technique was such that it could neither be utilized as a means of determination of estradiol in the presence of estrone, nor could a correction be made for the degree of augmentation of the estrone titer produced by its presence.

A modification has been found which gives similar colors with estrone and estradiol, estriol still remaining essentially negative. By this means, the total estrone and estradiol, as distinguished from other estrogens in a mixture, can be estimated in terms of estrone. Estradiol and estrone can then be separated by the Girard (2) reaction and the compounds estimated in the separate fractions.

Since concentrated sulfuric acid is present in the colorimetric reaction referred to, it is essential that all substances which will easily char with this reagent be eliminated from crude extracts before the analytical procedure can be applied. The impurities present in tissue extracts are mainly lipid extractives and non-hormonal chromogenic pigments. Since these

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differ from contaminants of urinary extracts, specific methods for dealing with them had to be found.

A combined set of procedures involving fractionation between immiscible solvents and chromatography has been developed which effects quantitative removal of interfering substances, permitting the colorimetric method to be applied to tissue extracts. Recovery of estrogens by this method, while not maximal, is of sufficient uniformity and reproducibility to render the techniques applicable to a study of estrogen metabolism in individual tissues.

### *Methods*

*Colorimetric Procedure*—The characteristics of the estradiol color in the original guaiacolsulfonic acid reaction were such that it appeared that the difficulty was due to incomplete conversion of the initial yellow chromogen of estradiol to the final pink obtained from estrone. By varying experimental conditions systematically, a satisfactory modification of the original method was developed, which gave pink colors of identical hue and similar intensity with both estrone and estradiol.

The following optimal conditions were developed for this modification. To each tube containing the dry hormone<sup>1</sup> 0.4 ml. of redistilled absolute ethanol is added. The wire rack of test-tubes is immersed in an ice and water bath and 2 ml. of concentrated sulfuric acid are added. Each tube is mixed with a footed stirring rod which is allowed to remain in the tube throughout the balance of the procedure. The tubes are placed in a boiling water bath for 1 minute. They are stirred once at the end of 30 seconds. On removal from the boiling water bath, the rack of tubes is replaced in the ice bath for 5 minutes. At the end of the cooling period, 4 ml. of a 7.5 per cent aqueous solution of potassium guaiacolsulfonate<sup>2</sup> are added. After thorough stirring, the rack is replaced for 2 minutes in the boiling water bath. The tubes are stirred once at the end of 30 seconds of the reheating procedure.

The rack of tubes is then placed once more in the ice bath and allowed to cool for a short period. After this, it is put in a water bath at 25° in the dark for 1 hour. At the end of this time, a final dilution with 3.6 ml. of 50 per cent sulfuric acid is made. 15 minutes later, the colors are read in an Evelyn photoelectric colorimeter. The standard 520 m $\mu$  filter supplied with the colorimeter was replaced by one with narrower spectral transmission to give a more selective range. This range was checked by a photoelectric spectrophotometer and found to be optimal.

<sup>1</sup> The crystalline hormones were kindly furnished by the Schering Corporation and by Hoffmann-La Roche, Inc.

<sup>2</sup> The potassium guaiacolsulfonate (thiocol) was generously supplied by Hoffmann-La Roche, Inc.

Strict observation of several conditions during the course of the reaction is required for proper results. (1) The water bath must be at a full boil during the heating stages. (2) All reagents are added while the tubes are in the ice bath in order to prevent much temperature rise on mixing (3). (3) Uniformity of the reaction mixture must be maintained at all stages during the reaction by standard mixing technique.

*Purification Procedures*—The following procedures constituted the routine method for isolation of estrogen from grossly impure tissue extracts.

1. *Hydrolysis*—The tissue-hormone sample in aqueous medium containing 20 per cent by volume of concentrated hydrochloric acid is hydrolyzed under a reflux on an electric hot-plate for 1 hour. This technique yields a relatively homogeneous mixture in which the tendency of emulsions to form during the subsequent chloroform extraction is minimal.

2. *Extraction with Chloroform*—The sample after hydrolysis, generally about 30 ml. in volume, is extracted four times with 15 ml. portions of chloroform. Thorough shaking for 2 minutes with each change of chloroform has been found adequate. Greaseless, water-lubricated stop-cocks are used in the separatory funnels throughout these procedures. The combined chloroform extract is filtered rapidly through chloroform-wet paper and evaporated to dryness on a steam bath in the presence of a small bead. Final traces of the solvent are removed *in vacuo*.

3. *Alcohol-Pentane Distribution*—Ralls *et al.* in 1926 (4) suggested partition of biological extracts containing fat and hormonal material between alcohol and petroleum ether, the latter being a much better solvent for lipids than for the estrogens. Koch<sup>3</sup> has also used this general procedure successfully. The dry material from step (2) is taken up in 20 ml. of 70 per cent ethyl alcohol and shaken against three 10 ml. portions of pentane, for 2 minutes in a 60 ml. separatory funnel. An occasional emulsion may be encountered which may be broken by violent shaking of the pentane phase after most of the clear alcohol has been drawn off, or by the addition of a drop of dilute hydrochloric acid to the pentane fraction followed by shaking. The alcohol phase is then evaporated to dryness in the presence of a bead. Small amounts of absolute alcohol may be added toward the end of this procedure to facilitate evaporation. A vacuum may be applied to remove the last traces of water vapor which may cling to the sides of the flask. Quantitative removal of fats is thus accomplished. A partial removal of pigment is also effected.

4. *Chromatographic Adsorption*—The residue from step (3) is taken up in 15 per cent acetone in pentane, as suggested by Heard and Hoffman (5). Both solvents must be redistilled. Any insoluble residue which remains in the sample flask is dissolved in water and extracted twice with 15 per cent

<sup>3</sup> Personal communication to L. T. S., June, 1940.

acetone-pentane (henceforth to be called the solvent), in order to remove the last traces of estrogen. This extract is added to the original solution.

The adsorption column consists for routine purposes of an ordinary Pyrex test-tube, 10 mm. inside diameter, 150 mm. long, fused to a short piece of glass tubing, beveled at the end, which serves as a stem. The column is plugged with a small amount of Corning glass wool. 4 gm. of activated<sup>4</sup> Merck's aluminum oxide, reagent grade, are weighed into it. The column must be cooled to prevent channeling due to boiling of the solvent during the subsequent procedure.

20 ml. of pure solvent are poured through the column and discarded. The sample extract is then poured through the column. This is followed by a total of 90 ml. of solvent, poured in small portions from the sample flask where it is used as a rinse. When the last drops of solvent have been blown out, the tip and inside of the stem of the column are washed with solvent, by means of a clean syringe and long needle, into the receiving flask. Pigment is retained in a narrow band at the top of the column.

The sample is evaporated to dryness at once with the aid of a bead in a warm water bath. The residue is in a white crystalline state, pure enough for colorimetry. In practice, at this point equal aliquots are removed to Pyrex test-tubes after solution of the sample in a known amount of absolute alcohol. One-half is used for direct colorimetry by the above method for total estrone and estradiol. The other aliquot is treated by a modified Girard procedure to obtain the non-ketonic fraction, estradiol, which is then determined by colorimetry as described.

The Girard procedure, partly adapted for micro quantities from Wolfe, Hershberg, and Fieser (6), is as follows: To the dry residue in a large test-tube, 0.5 ml. of a freshly prepared solution of Girard's Reagent T (100 mg. per ml. in glacial acetic acid which has been redistilled from potassium permanganate) is added. It is then stoppered with a glass marble and heated in a boiling water bath for 20 minutes, after which it is removed to an ice and water bath and thoroughly cooled. Dilution with 9.5 ml. of ice-cold water, followed by partial neutralization with 7.5 ml. of cold 1 N NaOH, is performed while the tube is still in the ice bath. The aqueous solution is transferred quantitatively to a 60 ml. separatory funnel. Three extractions with 10 ml. of pure chloroform are made. The combined chloroform extract representing the non-ketonic fraction is washed twice with 10 ml. of cold distilled water. It is then filtered through chloroform-wet paper, evaporated to dryness, and transferred with hot absolute alcohol to the test-tube for colorimetric analysis.

<sup>4</sup> Activation is performed for uniformity of adsorption characteristics by heating a quantity of pure aluminum oxide to red heat in an evaporating dish in a muffle furnace for 4 hours. On cooling, it is stored in tightly stoppered bottles in small lots.

## DISCUSSION

*Colorimetric Procedure*—Under the conditions described, estrone and estradiol give colors, the intensity of which varies directly with the concentration of hormone present. Fig. 1 demonstrates the applicability of Beer's law to samples containing from 10 to 40  $\gamma$ . Beyond approximately 40  $\gamma$  of estrone and slightly greater concentrations of estradiol, the absorption is less than theoretical. The relative intensity of the reaction in simultaneous

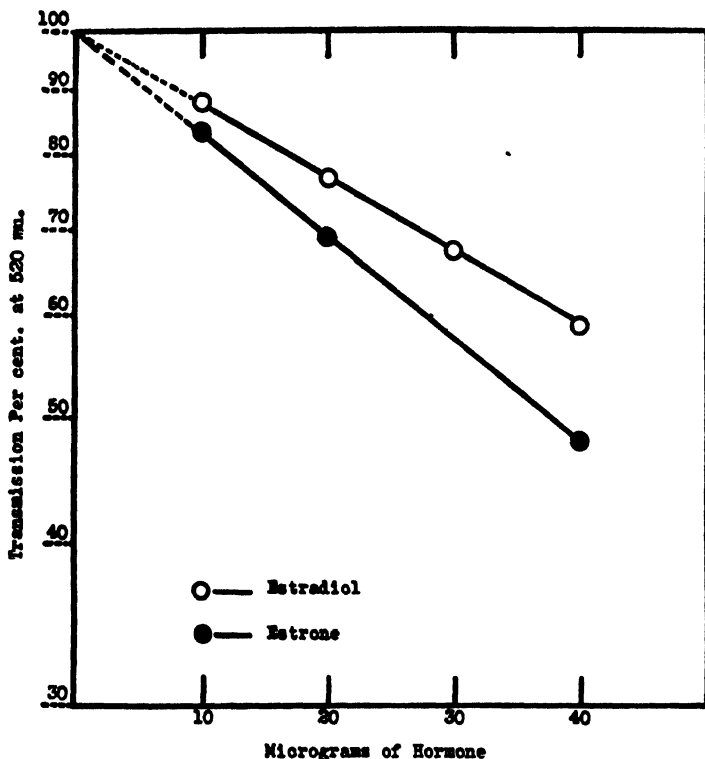


FIG. 1. Variation of per cent transmission with concentration in the revised guaiacolsulfonic acid reaction.

series of estrone and estradiol samples is also indicated by Fig. 1. Estrone invariably gave stronger color than estradiol under identical conditions.

Slight changes in slope of the calibration curves were observed from series to series. Individual conditions, probably affecting the degree of completeness of the yellow to pink color conversion, appear to exercise influence. Thus, it is apparent that a permanent calibration curve is incompatible with optimal accuracy. This difficulty may be overcome by including a set of

standards of both hormones in every series of unknowns, for the reproducibility of the reaction with each hormone is good within a single series. This is evidenced by the data in Table I showing the range of error in the determination of estrone and estradiol. The values in Table I were read from calibration curves based on standards run with each series. The correspondence is satisfactory. The range of error would have been significantly greater if all values had been determined from one composite calibration curve for each hormone. Duplicate determinations check well.

To study the stability of the color formed, a series of samples was read at intervals up to 17 hours. Significant fading did not take place at least within 1 hour after final dilution of the sample. Slight fading of the colors

TABLE I

*Range of Error in Determination of Estrone and Estradiol from Mean Curves for a Given Series*

No. of determinations*	Actual content		Found		Recovery
	Estrone	Estradiol	Estrone	Estradiol	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	<i>per cent</i>
1	5		4.9		98
6	10		10 -10.4		100-104
9	20		19.2-21.3		96-106
2	30		29.8-30.0		99-100
5	40		38.3-41.6		96-104
4		10		9.5-10.5	95-105
7		20		19.5-20.5	98-102
4		30		29.2-31.5	98-105
4		40		39.5-40.0	99-100

\* Not necessarily simultaneous series.

did occur on longer standing. This is not a serious consideration in routine determinations.

Table II demonstrates the applicability of this revised guaiacolsulfonic acid method to known mixtures of estrone and estradiol. When standard estrone and estradiol samples, singly, are included with a series of mixtures of the two, the chromogenic effect of given amounts of estradiol may be calculated from the calibration curves for the individual hormones in terms of estrone.

Two things are apparent from Table II: first, that additivity of the chromogenic effect of the two hormones exists in a mixture; second, that standards of the individual compounds, estrone and estradiol, at one concentration level may be used to set the reference curves for the whole series. In other words, the reaction for the individual hormones alone may be used as a standard for the intensity and additivity of the reaction in a mixture within a single series.

An advantage of the revised guaiacolsulfonic acid method in common with the original adaptation (1) is the failure of estriol to react appreciably with the color reagent. This is seen in Table III which shows the colorimetric effect of varying amounts of estriol. Quantities of the order of 10 to 20  $\gamma$  (at which level the reaction is well suited to the estimation of estrone and estradiol) have negligible colorimetric effect. With larger amounts of estriol, to 40  $\gamma$ , a small increment of colorimetric reading is observed. In no case was an actual pink color apparent to the eye. The error would

TABLE II  
*Additivity of Mixtures of Estrone and Estradiol*

Series and sample No.	Actual content		Calculated total content as estrone*	Found	Recovery
	Estrone	Estradiol		Estrone	
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
1a	5	5	9.2	9.2	100
1b	5	10	13.5	14.0	104
1c	5	20	22.5	24.1	107
1d	10	10	18.5	19.1	103
1e	20	20	37.5	40.7	108
2a	5	20	20.0	20.0	100
2b	5	20	20.0	20.8	104
2c	5	30	27.8	27.0	97
2d	5	30	27.8	27.5	99
3a	10	20	24.8	25.0	101
3b	10	20	24.8	24.8	100
3c	15	15	26.2	26.6	102
3d	15	15	26.2	26.8	102
3e	20	10	27.4	27.8	101
3f	20	10	27.4	27.8	101
4a	15	15	24.0	23.7	99
4b	15	15	24.0	23.9	99
5	10	20	26.0	26.0	100

\* These figures are based on standards of estrone and of estradiol run with each series. The slight variation in the calibration curves from series to series as described in the text accounts for the different calculated total estrone values for the same mixture in different series.

be significant in the presence of the large proportion of estriol found in urine extracts. In this case a preliminary partial separation should be made (7). Because of other difficulties in purification, however, the colorimetric method has not yet been applied successfully to this biological fluid.

On longer initial heating, estriol reacts to give a pink of moderate intensity under the original conditions of the guaiacolsulfonic acid method. Similarly, with this modification, a 20 minute initial heating time gives a pink color with estriol without parallel change in intensity for estrone and



estradiol. The possible conversion of the 16-17 hydroxy compound to estrone under dehydrating conditions has been pointed out (1).

TABLE III  
*Colorimetric Effect of Estriol*

Sample No.	Readings at 520 mμ	Actual estriol content	Found, equivalent to	
			Estrone	Estradiol
		γ	γ	γ
1	100	10	0	0
2	100	10	0	0
3	99.00	20	0.5	0.7
4	98.00	20	1.1	1.4
5	95.75	30	2.6	3.0
6	95.00	30	3.0	3.5
7	95.25	40	2.9	3.4
8	95.50	40	2.7	3.2

TABLE IV  
*Recovery of Pure Estrone from Several Steps of Purification Procedure*

Series and sample No	Estrone added	Added* before	Estrone found	Recovery	Loss in step involved
	γ		γ	per cent	per cent
1a	25	Chromatography	25.2	101	
2a	25	"	25.8	103	0
3a	25	Alcohol-pentane distribution	23.0	92	
4a	25	" "	24.0	96	8
5a	25	CHCl <sub>3</sub> extraction	23.4	93	
6a	25	" "	22.6	91	2
7a	25	Hydrolysis	20.1	80	
8a	25	"	20.2	81	12
Total .....					22
1b	30	Chromatography	26.8	89	11
2b	30	Alcohol-pentane distribution	24.5	82	7
3b	30	CHCl <sub>3</sub> extraction	23.0	77	5
4b	30	Hydrolysis	20.5	68	
5b	30	"	22.0	73	6
Total					29

\* All samples were run in parallel from the start of the purification procedure regardless of time of addition of estrone.

Neither androsterone, progesterone, nor cholesterol gives the reaction. While these substances are ordinarily separated from the estrogens in the

TABLE V  
*Recovery of Pure Hormones from Complete Purification Procedure*

	Series and sample No	Added			Found						
		Estrone	Estradiol	Total as estrone	Total			After Girard separation			
					As estrone	As estradiol		Non-ketonic as estradiol*		Ketonic, by difference	
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent
Estrone	1	25			13.5		54				
	2a	25			20.1		80				
	2b	25			20.2		81				
	3a	30			20.5		68				
	3b	30			22.0		73				
	4	40			22.0		55				
	5a	40			22.5		56				
	5b	40			21.8		55				
Average. . . . .							65				
Estradiol	1a	25				13.0	52				
	1b	25				13.2	53				
	2	40				27.0	68	22	55		
	3a	40				20.5	51				
	3b	40				24.0	60	19.5	49		
	4a	40				23.8	60	16.5	41		
	4b	40				21.5	54	15.0	38		
Average . . . . .							57		46		
Mixtures	1	20	20	31.8	17.3		54	9.6	48	10.8	54
	2a	12.5	12.5	21.5	15.5		72	9.5	76	9.2	73
	2b	12.5	12.5	21.5	15.5		72	10.0	80	8.8	70
	3a	24	16	36	18.5		52	9.5	59	12.9	54
	3b	24	16	36	20.5		57	11.0	69	13.7	57
	3c	16	24	34	19.5		57	15.8	66	9.9	62
	3d	16	24	34	17.5		52	13.0	54	9.5	59
	4a	24	16	36	21.5		60	7.0	44	16.3	68
	4b	24	16	36	21.5		60	7.0	44	16.3	68
	4c	16	24	34	20.0		59	10.0	42	12.4	78
	4d	16	24	34	20.0		59	10.0	42	12.4	78
Average.....							59		57		66

\* Corrected for 3  $\gamma$  of total reagent blank.

preparation of biological extracts, it is advantageous that small amounts carried through into the estrogen fraction will not interfere with the analysis by this method.

*Purification Procedures*—Table IV summarizes the results of two experiments in which a known quantity of estrone was added to samples at a

TABLE VI  
*Recovery of Pure Hormones in Presence of Boiled Tissue\**

	Series and sample No.	Hormone added	Total found	Corrected for tissue	
		$\gamma$	$\gamma$	$\gamma$	per cent
Estrone	1	25	17.0	14.5	58
	Tissue blank	0	2.5	0.0	
	1a	25	17.5	14.0	56
	Tissue blank	0	3.5	0.0	
	2a	40	26.0	23.8	60
	2b	40	26.0	23.8	60
	Tissue blank	0	2.2	0.0	
	3a	30	28.5	21.7	72
	3b	30	23.5	16.7	56
	Tissue blank	0	6.8	0.0	
	4a	25	10.8	10.3	41
	4b	25	11.7	11.2	45
	Tissue blank	0	0.5	0.0	
	5a	25	14.9	11.6	46
	5b	25	15.5	12.2	49
	Tissue blank	0	3.3	0.0	
	6a	25	17.3	15.8	63
	6b	25	16.1	14.6	58
	6c	25	14.3	12.8	51
	Tissue blank	0	1.5	0.0	
	7a	25	19.6	16.7	67
	7b	25	15.1	12.2	49
	Tissue blank	0	2.9	0.0	
Average. . .					55
Estradiol	1	25	22.3†	17.8	71
	Tissue blank	0	4.5†	0.0	
	2a	40	30.5†	26.5	66
	2b	40	30.5†	26.5	66
	Tissue blank	0	4.0†	0.0	
Average . . .					68

\* Endometrium, cow, human, rabbit.

† As estradiol.

definite stage of the purification procedure, the samples being carried as complete reagent blanks up to the point of addition of the hormone. It is apparent that each step of the procedure involves a definite loss of estro-

gen, owing to handling or possible destruction. Nevertheless, duplicate samples appear to check well, although not necessarily from series to series.

Table V shows the recovery of estrone and estradiol, alone and in mixtures, from the complete purification procedure. While the variation of the error in recovery from series to series appears to be relatively large, and the magnitude of the error itself considerable, it will be noted that within a single series, as indicated by the numbering of the samples, recoveries are fairly uniform and consistent. In our experience this reproducibility of recovery within each experiment has served as a reliable correction factor. It appears to be independent of the amount of estrogen initially present, and, as is evident from Table VI, independent of the presence of tissue, or, within limits, of the amount thereof.

The possible sources of the error in recovery appear to be the following: (1) Some destruction during hydrolysis. This is independent of hydrolysis up to 1 hour, however. (2) Losses in slight emulsions formed during the extraction of the hydrolysate with chloroform. Emulsions form to a larger extent if the hydrolysis step is omitted, as would be expected. (3) Some slight solubility of estrogen in pentane probably accounts for the losses in the alcohol-pentane distribution step, which, however, is essential for complete removal of interfering lipids. (4) The losses involved in chromatography are generally due to incomplete washing of the column, especially in hot weather, when evaporation becomes a large factor. It has been found that under these conditions washing the column with 30 ml. of 15 per cent acetone-pentane after it has dried from the preceding experiment will occasionally cause elution of some further estrogen.

The losses in each step appear to be variable from experiment to experiment, but the over-all recovery in each series is quite uniform. Therefore a pure standard without tissue may be run with each series in order to compensate for the over-all loss in the experiment. The average recovery of all the experiments reported is 59.6 per cent.

#### SUMMARY

A color reaction suitable for the determination of total estrone and estradiol without significant interference from estriol has been described. The specificity, accuracy, and sensitivity of the analytical procedure are satisfactory.

This colorimetric method has been successfully applied to estrone and estradiol recovered from addition to tissue samples by means of the isolation and purification procedures developed. The recoveries from the purification procedures, while not maximal, are of sufficient uniformity and consistency within each series to permit a reliable correction.

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# THE METABOLISM OF ESTRONE IN SURVIVING RABBIT, BOVINE, AND HUMAN ENDOMETRIUM\*

BY CLARA M. SZEGO AND LEO T. SAMUELS

(From the Divisions of General Physiology and Physiological Chemistry, Department of Physiology, University of Minnesota Medical School, Minneapolis)

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The conversion of estrone to estradiol by uterine tissue of the rat and rabbit was postulated by Heller and his associates (1-3). The evidence presented for this conclusion was the increase in estrogenic potency observed after incubation of the tissue with estrone.

Since the technique of biological assay used does not permit of quantitative interpretations in unknown estrogen mixtures, a study of the problem by more specific methods of identification was thought desirable. Chemical methods (4) developed in this laboratory which enabled us to analyze tissues and distinguish estrone and estradiol were found to be applicable to this problem. The possible effect of uteri of other species was also investigated.

## Materials and Methods

The endometria used in this study were bovine, pregnant human, and pregnant rabbit. They were obtained in the following manner.

*Bovine*—The uterus was removed at the slaughter-house immediately after the death of the animal. After its healthy condition had been checked by the government veterinarian, it was rushed in a clean container surrounded by ice to the laboratory, a trip consuming about 10 minutes.

*Human*—At the University Hospital a therapeutic hysterectomy was performed on a woman who was 3 months pregnant. The uterus was taken to the laboratory for the incubation experiment within 5 minutes of removal.

*Rabbit*—The animal was killed by a blow on the head, bled from the throat, and the uterus immediately removed.

The endometrium was rapidly dissected as free as possible from the underlying tissue, and removed in sections with scrupulously clean, sharp instruments. Excess moisture was blotted off gently with filter paper. Samples of endometrium, and in some cases, ovary, from each experi-

\*A portion of the data in this paper was taken from a thesis submitted by C. M. Szego to the Graduate School of the University of Minnesota in August, 1942, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ment were preserved in formalin for later sectioning. These were examined by Dr. Willard Boyd of the Division of Veterinary Medicine to determine the stage of the estrous cycle.

Approximately 1 to 2 gm. samples were removed to a clean cork, and either sliced perpendicularly to the surface or finely minced, with a specially designed instrument. This device consisted of ten to twelve thin razor blades clamped parallel to the long axis of a metal holder, slightly less than 0.2 mm. apart. This thickness of tissue slices has been considered compatible with normal respiratory function as measured by the Warburg technique (5).

The minced or sliced tissue was weighed rapidly on a pharmaceutical balance and washed into the incubation flask with 25 ml. of the liquid medium. Estrone or estradiol was added in not more than 0.25 ml. of 95 per cent ethanol. When destruction of the living tissue was desired, it was washed into a flask in which a portion of the liquid medium had been brought rapidly to a boil, and was then further boiled for a short time.

The medium consisted of water 12 parts; Locke's stock solution, 3 times concentrated (22.8 gm. of NaCl, 0.13 M; 0.447 gm. of KCl, 0.002 M; 0.333 gm. of  $\text{CaCl}_2$ , 0.001 M in 1 liter), 8 parts; 0.2 M sodium phosphate buffer adjusted to pH 7.4 (200 ml. of 0.2 M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  mixed with 800 ml. of 0.2 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) 2.4 parts; freshly prepared 0.16 M glucose 1.6 parts. Triple, glass-distilled water was used throughout.

As soon as the tissue was added, each flask was stoppered with a glass stopper and clamped onto a spoke radiating from a motor-driven axle in an incubator tank of approximately 50 gallons capacity. The temperature of the bath was thermostatically controlled at 38.5° for the bovine, 37° for the human, and 40° for the rabbit tissues,  $\pm 0.5^\circ$ , respectively. The time from the start of the dissection procedures to the beginning of the incubation averaged about 15 minutes when eight flasks were used. The air-filled flasks were incubated with constant rotation (about thirty per minute) for 1 or 2 hour periods.

At the end of the incubation period, the samples were removed and 6 ml. of concentrated hydrochloric acid were added to each. The material was immediately hydrolyzed by boiling for 1 hour and the resultant mixture extracted and purified as described elsewhere (4).

The estrogens so isolated from the incubation mixture were then divided into equal aliquots. One aliquot was analyzed for total estrogens as estrone. The non-ketonic fraction of the second aliquot was separated by a modified (4) Girard procedure. It was then analyzed for estradiol. The colorimetric procedure used was the guaiacolsulfonic acid method (4) previously described.

In several experiments, samples of the same endometrium were subjected

to a simultaneous study of oxygen consumption by the Warburg technique. The medium in these cases was the same as that in the incubation studies, a 3 ml. volume being used. The gaseous phase was air.<sup>a</sup>

### Results

*Bovine Endometrium*—As shown in Table I, there was striking similarity between the recoveries of total estrogen from boiled and from fresh tissue. If the percentage recovery of total estrogen from fresh tissue is tabulated for each experiment, the recovery from the corresponding boiled tissue samples being taken as 100 per cent, the average with its standard error is 99.5 per cent  $\pm$  1.4.

TABLE I

*Relation of Stage of Estrous Cycle to Recovery of Ketonic and Non-Ketonic Material after Incubation with Living Tissue*

Stage of estrous cycle	Estrone added	Average recovery in ketonic fraction		Average recovery in non-ketonic fraction as "estradiol"†
		Estrone	Per cent of boiled tissue*	
	$\gamma$	$\gamma$		$\gamma$
Estrus	30	18.1	95.0	
Preestrus	50	21.8	101.5	0.5
7-8 days post estrus. . . .	50	23.9	100.1	1.8
Advanced post estrus	50	29.7	101.4	0.5

\* Calculated on the basis of the recovery after addition of similar amounts of hormone to boiled tissue controls, in each series, taken as 100 per cent.

† A mean of 18  $\gamma$  of non-ketonic material was recovered from boiled tissue when 50  $\gamma$  of estrone were added.

The mean percentage of added estrogens recovered in the analyses, expressed as estrone, was 55.5 per cent with a standard error of  $\pm$ 1.8 per cent. This is not significantly different from the recovery of pure hormones carried through the purification procedure (4). There does not appear to be any large amount of destruction of the hormones in the presence of viable endometrium.

The apparent recovery of non-ketonic material from incubation of bovine endometrium with estrone was insignificant. The recovery from boiled tissues averaged 1.78  $\gamma$  (0.76 to 3.92) and that from fresh tissues 0.83  $\gamma$  (0.46 to 1.81). However, the reagent blanks for the entire purification procedures gave small measurable readings for the non-ketonic fraction. The tissue controls without added estrogen, moreover, gave colorimetric readings for the non-ketonic fraction which were of the same order of magnitude as in the samples with added estrone. Some contaminant carried



over from the Girard treatment apparently accounts for the small but consistent readings for "estradiol."

As seen in Table I, tissues were obtained during all the major phases of the estrous cycle. There was no evidence of conversion or greater destruction during any particular phase.

Warburg studies on oxygen consumption of samples of the same endometrial tissue were run simultaneously with several experiments. The tissues continued to consume oxygen for the 100 minute period during which measurements were made. The  $Q_{O_2}$  (90 minutes) of minced tissue averaged 4.3, and that of the sliced tissue 2.2. The steeper slope observed with the minced tissue is apparently due to the larger surface with this state of subdivision. These data indicate that the tissues were probably viable and showed active metabolism over at least 2 hours.

*Human Endometrium*—The data from the treatment of human endometrium obtained as described from a pregnant uterus fall in line with the observations on the non-pregnant bovine tissue. The recoveries of total estrogen as estrone from boiled and fresh tissue samples were similar, and analysis of the non-ketonic fractions gave a small negative value when corrected for tissue blanks. Oxygen consumption of samples of this tissue gave somewhat higher absolute values for the 90 minute period than did those of the non-pregnant bovine tissue (average  $Q_{O_2}$  (90 minutes) = 6.51).

*Rabbit Endometrium*—The results of incubation experiments with tissue from two pregnant rabbits are summarized in Table II. The data indicate clearly that conversion of estrone to estradiol takes place in this tissue. The evidence for this conclusion lies primarily in the fact that there is a considerably higher non-ketonic recovery from the samples of fresh tissue compared with the boiled tissue after incubation with estrone. There is also no significant difference between Sample 4a and Samples 5a to 8a, although the proportions of estrone and estradiol added to the fresh tissues varied. This suggests a quantitative conversion of estrone to estradiol in the fresh tissues in this experiment.

In the second experiment of this series, twice as much hormone was used as in the earlier one, with similar amounts of tissue. Samples 1b to 4b, representing equivalent amounts of estrone and estradiol added to the incubation mixture with fresh tissue, show a high recovery of non-ketonic material which does not differ significantly among the four samples. The recovery of ketonic material after the addition of estrone is much greater and the non-ketonic fraction much smaller in the boiled tissues (Samples 5b, 6b). Apparently estrone was converted to estradiol in pregnant rabbit endometrium by a mechanism which was destroyed by boiling.

Since the recoveries of ketonic material are larger in the fresh tissues of Series b than in the boiled tissues to which estradiol was added, while the

reverse is true for the non-ketonic fraction, it would appear that the conversion of 100  $\gamma$  of estrone to estradiol was not complete. This seems to be due to the establishment of an equilibrium, since the fresh tissues to which estradiol had been added (Samples 3b, 4b) show a recovery of ketonic material similar to the estrone samples (Nos. 1b, 2b) and greater than the amounts found in the presence of boiled tissue (Samples 7b, 8b).

An additional observation to be made from these experiments is that recovery of estradiol is satisfactory from both fresh and boiled tissue, confirming previous data (4). This indicates that failure to observe incre-

TABLE II

*Recovery of Estrone and Estradiol from Incubation with Pregnant Rabbit Endometrium*

Sample No.	State of tissue before incubation	Estrone added	Estradiol added	Found*	
				Non-ketonic as estradiol	Ketonic (by difference) as estrone
		$\gamma$	$\gamma$	$\gamma$	$\gamma$
1a	Boiled			0.0	0.0
2a	"	50		5.0	25.8
3a	"	50		8.0	22.7
4a	"		50	22.0	7.0
5a	Fresh	50		23.0	8.2
6a	"	50		22.0	6.0
7a	"		50	28.0	4.0
8a	"	25	25	22.0	7.0
1b	"	100		45.5	13.5
2b	"	100		43.0	21.3
3b	"		100	45.3	14.7
4b	"		100	45.8	21.4
5b	Boiled	100		7.5	54.5
6b	"	100		7.0	54.6
7b	"		100	61.7	7.5
8b	"		100	58.0	9.6
9b	Fresh			0.0	0.0

\* Calculated on the basis of the total sample. Corrected for tissue blanks.

ments of chromogen in the non-ketonic fractions in the experiments with bovine and human uterine tissue was not due to selective loss of estradiol in the purification procedures.

#### DISCUSSION

The evidence presented above indicates that the non-pregnant bovine and pregnant human uteri do not possess the mechanism for the conversion of estrone to the more active estradiol postulated by Heller and his associates (1-3). On the other hand, the experiments here reported show that such conversion does take place in the endometrium of the pregnant rabbit.

There appears to be a difference between species, and no interspecies generalization can be made.

The uniformity of recovery of total estrogen measured as estrone from boiled and fresh endometria of the bovine and human samples, as well as failure to find significant differences in the non-ketonic fractions, weighs heavily against the possibility of conversion in these species. Such conversion would have been recognized in two ways by our methods. (1) Recovery of total estrogens would have been consistently decreased in the boiled tissue, for estradiol has a significantly lower colorimetric effect than has estrone by the analytical method used (4). (2) Recovery of estradiol in the non-ketonic Girard fraction would have been consistently increased in the fresh tissue over that of the boiled. These criteria were not fulfilled in the case of human and bovine endometria.

It may be argued that small differences would be missed by use of the colorimetric method for identification of the active substances. While this may be true for quantities of the order of 1 to 3  $\gamma$ , the method is sufficiently sensitive for the recognition of conversion by the criteria mentioned above beyond these minute quantities. Moreover, Heller reported (2) complete conversion of at least 1.5  $\gamma$  of estrone to estradiol by 250 mg. of tissue in 1 hour. On the scale of incubation time and tissue weights used in our experiments, this would have involved conversion of at least 12  $\gamma$ , a change which would not have been missed by our techniques.

The results on the human tissue are in agreement with the observation of Twombly and Taylor (6) who found no augmentation of the physiological activity of estrone incubated with human endometrial curettings.

On the other hand, the experiments herein described give ample evidence of conversion of estrone to estradiol in rabbit endometrium, a finding which gives added weight to the negative results with the other species. Both the Heller and Twombly groups have reported conversion of estrone incubated with rabbit tissue on the basis of increased potency by biological assay. Bioassay of crude tissue extracts, however, is subject to the criticism that the hormonal activity is modified by inert contaminants (7). Actual isolation of the formed estradiol and identification by physical constants would be extremely difficult with quantities of the order of micrograms. Short of this, the best available means of identification of estradiol as a possible conversion product of estrone would appear to be isolation and measurement by a chemical reaction possessing good specificity.

The data herein reported, therefore, throw serious doubt upon a conversion, mole for mole, of estrone to estradiol in surviving endometrium of the bovine and human species. Such a mechanism, however, has been shown to exist in the endometrium of the pregnant rabbit under identical conditions.

## SUMMARY

Samples of viable endometrium from the bovine during various stages of the estrous cycle, from a woman 3 months pregnant, and from pregnant rabbits were incubated aerobically with estrone and estradiol.

The bovine endometrium neither destroyed estrone significantly nor converted it to estradiol during any stage of the estrous cycle. This was also true of the endometrium of the pregnant woman.

The endometrium of the pregnant rabbit appeared to convert estrone almost entirely to estradiol.

The authors wish to express their indebtedness to Mrs. C. J. Carter for the Warburg studies.

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# ENZYMATIC HYDROLYSIS OF URINARY SODIUM PREGNANEDIOL GLUCURONIDATE TO FREE PREGNANEDIOL\*

By NATHAN B. TALBOT, JOSEPHINE RYAN, AND JOHN K. WOLFE

*(From the Department of Pediatrics, Harvard Medical School, and the Children's Medical Service, Massachusetts General Hospital, Boston)*

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The available evidence suggests that most of the steroid hormone end-products which are eliminated from the body by way of the urine are conjugated with an inorganic or organic acid. This conjugation presumably facilitates their transportation and excretion by rendering them soluble in water or urine. Examples of such conjugated steroids which have been isolated from urine as sulfates are androsterone (1), dehydroisoandrosterone (2), and estrone (3), and as glucuronidates, estriol (4) and pregnanediol (5). Convenient methods for measuring the rate of urinary excretion of these conjugated substances depend to a major extent upon hydrolysis of the conjugated steroids to unconjugated steroids. Most of the latter are essentially insoluble in water and can be extracted from aqueous solutions with suitable organic solvents.

In a previous paper (6) evidence was presented which showed that the commonly employed hydrochloric acid hydrolysis procedure tends to destroy dehydroisoandrosterone added to water as sodium dehydroisoandrosterone sulfate. On the other hand, it was found that the sodium dehydroisoandrosterone sulfate could be hydrolyzed without losses of dehydroisoandrosterone by barium chloride solution at pH 5. Evidence is available which shows that hydrochloric acid hydrolysis of sodium pregnanediol glucuronide also is accompanied by an approximately 30 per cent loss of pregnanediol (7). However, trial experiments have revealed that barium chloride does not hydrolyze sodium pregnanediol glucuronide.

The possibility that sodium pregnanediol glucuronide could be hydrolyzed satisfactorily with the aid of glucuronidase was suggested by the observations of Fishman (8). He found that an enzyme obtained from beef spleen tended to hydrolyze borneol glucuronide and certain other glucuronidates. Because beef spleen enzyme prepared in this laboratory according to Fishman's directions failed to hydrolyze sodium pregnanediol glucuronide to pregnanediol satisfactorily, the use of an enzyme prepared from rat liver according to the directions of Astwood<sup>1</sup> was investigated.

\* This work was supported by a grant from the Commonwealth Fund of New York.

<sup>1</sup> Astwood, E. B., unpublished observations.

The present paper shows that this rat liver enzyme hydrolyzes sodium pregnanediol glucuronidate in pure aqueous solutions and in aqueous solutions of *n*-butanol extracts of urine to free pregnanediol in good yields.

### *Analytic Procedure*

*Preparation of Enzyme*—Approximately six strictly fresh livers from young adult rats are obtained. These are ground into a paste with mortar and pestle. Acetone, c.p., is added in small quantities and the grinding continued until the liver is finely divided. 10 volumes of acetone are then added with stirring. After standing 15 minutes the supernatant acetone is decanted and an equal volume of fresh acetone is added, again with stirring. When the mixture has stood for another 15 minutes, it is filtered on a Buchner funnel with the aid of moderate suction. The residue on the filter is washed three times with small quantities of anhydrous ether and sucked dry. As much as possible of the resulting dry residue is sifted through a tea strainer. The finely divided powder which passes through the strainer contains the enzyme. This dry, defatted liver powder can be stored in a stoppered, dark bottle at ice box temperatures for at least a month without appreciable loss of activity.

*Extraction of Sodium Pregnanediol Glucuronidate from Urine*—Urine is collected with 200 cc. of *n*-butanol per liter added as a preservative at the beginning of the collection period. A washed and dried *n*-butanol extract of the urine is prepared according to directions outlined elsewhere (6).

*Enzymatic Hydrolysis of Sodium Pregnanediol Glucuronidate*—The residue of a dried, *n*-butanol extract from an approximately 24 hour sample of urine is dissolved in 15 cc. of distilled water.<sup>2</sup> 10 cc. of 0.1 *N* sodium acetate buffer (adjusted to pH 5.0) (9) and 400 mg. of the rat liver powder are then added. The reaction mixture thus prepared is incubated for 4 hours at 37°.

*Recovery and Colorimetric Assay of Free Pregnanediol*—During the period of incubation, the water-soluble pregnanediol glucuronidate is hydrolyzed to free pregnanediol which is insoluble in water. The incubated reaction mixture is chilled and then filtered through Whatman No. 2 filter paper. The residue, which contains the free pregnanediol, is washed on the filter three times with 5 cc. portions of cold water. The combined filtrate and water washings are discarded.

The free pregnanediol contained in the precipitate is then extracted from the precipitate by elution at least four times with 5 cc. lots of hot 95 per cent ethanol. The combined alcoholic extract is evaporated to dryness by heating in a boiling water bath. The residue is dissolved in 10 cc. of hot

<sup>2</sup> If the residue of the butanol extract is not soluble in 15 cc. of water, proportionately larger quantities of water and reagents may be used.

95 per cent ethanol and the pregnanediol is precipitated from the solution by the slow addition of 40 cc. of water as described elsewhere (7). After the precipitated pregnanediol has been collected, it is assayed colorimetrically (10).

#### EXPERIMENTAL

*Sodium Pregnanediol Glucuronide*—In these experiments, two samples of this substance were used. The first, Sample A, was crystalline sodium pregnanediol glucuronide (m.p. 270–272°) obtained through the courtesy of Ayerst, McKenna and Harrison, Ltd. The second, Sample B, was prepared from Sample A by repeated recrystallization from alcohol-water solutions. Sample B had a melting point of 279–280° and a sodium content of 4.3 per cent (theoretical for  $C_{27}H_{48}O_8NaH_2O$ , 4.3 per cent). The mixed

TABLE I

*Relation between pH of Reaction Mixture and Yield of Free Pregnanediol Following Enzymatic Hydrolysis of a Constant Quantity of Sodium Pregnanediol Glucuronide (Sample A) Substrate*

Experiment No.	pH*	Pregnanediol recovered
		mg.
1	1	0
2	2	1.4
3	3	1.5
4	4	2.1
5	5	2.2
6	6	2.1
7	7	1.6

\* Adjustment of pH values was accomplished with one of the following approximately 0.1 N buffer solutions: pH 1, citric and hydrochloric acids; pH 2 and 3, disodium phosphate and citric acid; pH 4 and 5, sodium acetate; pH 6 and 7, sodium phosphate.

melting point with an authentic sample of sodium pregnanediol glucuronide (m.p. 279–280°) was 279–280°.

*Conditions of Hydrolysis*—The optimal pH was determined as follows: To 5 cc. lots of an aqueous solution containing approximately 5 mg. of sodium pregnanediol glucuronide (Sample A) and 150 mg. of rat liver enzyme powder were added 5 cc. of approximately 0.1 N citrate, acetate, or phosphate buffer solutions with respective pH values from 1 to 7. These mixtures of different pH were then incubated for 24 hours at 37°. The quantity of free (hydrolyzed) pregnanediol was determined as in the analytic procedure described above. The results (Table I) show that the yield of free pregnanediol was greatest when the incubation was carried out at pH 5.



To determine the length of time necessary for hydrolysis at the optimal pH, mixtures similar to that used in Experiment 5 of Table I were incubated at 37° for varying periods of time. The results given in Table II demonstrate that the yield of hydrolyzed pregnanediol reached a maximum at approximately 3 hours. Longer incubation neither increased nor decreased the yield.

TABLE II

*Relation between Duration of Enzymatic Hydrolysis, at pH 5 and Yield of Free Pregnanediol from a Constant Quantity of Sodium Pregnanediol Glucuronidate (Sample A) Substrate*

Experiment No.	Duration of hydrolysis	Pregnanediol recovered
	<i>hrs.</i>	<i>mg.</i>
8	$\frac{1}{4}$	0
9	$\frac{1}{2}$	0.3
10	1	1.1
11	3	1.8
12	8	1.8
13	19	1.7
14	24	1.8

TABLE III

*Relation between Concentration of Enzyme in Hydrolysis Mixture (Incubated 4 Hours at pH 5) and Yield of Pregnanediol from a Constant Quantity of Sodium Pregnanediol Glucuronidate (Sample A) Substrate*

Experiment No.	Concentration of enzyme	Pregnanediol recovered
	<i>mg. per cent</i>	<i>mg.</i>
15	0	0
16	80	0.1
17	165	0.6
18	420	1.0
19	800	1.6
20	1,650	1.7
21	8,000	1.7
22	16,500	1.7

The concentration of enzyme necessary for optimal hydrolysis was investigated by adding to 5 cc. of an aqueous solution containing approximately 5 mg. of sodium pregnanediol glucuronidate (Sample A), 5 cc. of 0.1 N sodium acetate buffer (pH 5), and various quantities (8 to 1650 mg.) of the enzyme powder. The resultant mixture was incubated for 4 hours at 37°. The results (Table III) reveal that a maximum yield was obtained

when the enzyme concentration was 1650 or more mg. per cent. When enzyme was added to make a 10-fold higher concentration (16,500 mg. per cent), identical results were obtained.

*Quantitative Efficiency of Enzymatic Hydrolysis Procedure*—Table IV shows the quantity of free pregnanediol recovered from aqueous solutions containing measured quantities of analytically pure sodium pregnanediol glucuronidate (Sample B) hydrolyzed according to the analytic procedure. The theoretical yield of free pregnanediol from 1 mg. of sodium pregnanediol glucuronidate is 0.597 mg. It is seen that the average recovery of free pregnanediol was 89 per cent of the theoretical yield (range 86 to 92 per cent).

The combined hydrolysates of these three experiments melted at 224–226°. After crystallization from ether the melting point was 235–236°.

TABLE IV

*Recovery of Pregnanediol Following Enzymatic Hydrolysis of Pure Sodium Pregnanediol Glucuronidate (Sample B) under Optimal Conditions (pH 5 for 4 Hours at 37° with an Enzyme Concentration of 1600 Mg. Per Cent)*

Experiment No.	Sodium pregnanediol glucuronidate added	Pregnanediol recovered		
		Theoretical* (a)	Determined (b)	$\frac{(b)}{(a)}$
	mg.	mg.	mg.	
23	None	0.0	0.0	
24	10.0	6.0	5.5	0.92
25	4.7	2.8	2.4	0.86
26	4.2	2.5	2.2	0.88

\* The theoretical yield of free pregnanediol from 1 mg. of sodium pregnanediol glucuronidate is 0.597 mg.

The melting point of this material when mixed with an authentic sample of pregnanediol (m.p. 236–237°) was 236–237°. This indicates that the material recovered after enzymatic hydrolysis of sodium pregnanediol glucuronidate was pregnanediol.

*Efficiency of n-Butanol Extraction of Sodium Pregnanediol Glucuronidate (Sample A) Added to Urine*—The efficiency with which pregnanediol, added to urine as sodium pregnanediol glucuronidate, could be recovered by the analytic procedure outlined above was investigated as follows: 150 to 1000 cc. lots of freshly voided urine were obtained. Each lot was divided into two equal portions. To one aliquot nothing was added; to the other a measured quantity of an aqueous solution of sodium pregnanediol glucuronidate (Sample A) was added. Because the available sodium pregnanediol glucuronidate was not chemically pure, the concentration of this

substance in the aqueous solution added to urine was determined by direct enzymatic hydrolysis followed by colorimetric assay according to the analytic procedure. Thus, though the losses inherent in the enzymatic hydrolysis and colorimetric assay portion of the analytic procedure are canceled out in these experiments, they do give accurate information concerning the efficiency of the *n*-butanol extraction portion of the procedure.

The data obtained (Table V) show that the pregnanediol recovered following the addition of sodium pregnanediol glucuronide to urine was on the average 92 per cent (range 79 to 100 per cent) of the theoretical

TABLE V

*Recovery of Pregnanediol Added to Urine As Sodium Pregnanediol Glucuronide (Sample A) Prior to n-Butanol Extraction and Enzymatic Hydrolysis*

Experiment No.	Sodium pregnanediol glucuronide added*	Pregnanediol recovered		
		Theoretical† (a)	Determined (b)	$\frac{(b)}{(a)}$
	mg	mg.	mg	
27a	0		0	
27b	3.0	1.8	1.7	0.94
28a	0		0	
28b	3.0	1.8	1.8	1.00
29a	0		1.0	
29b	3.0	2.8	2.7	0.96
30a	0		0	
30b	3.0	1.8	1.4	0.79

\* A measured quantity of a stock, aqueous solution of sodium pregnanediol glucuronide (Sample A) was added in these experiments. The concentration of pregnanediol (as sodium pregnanediol glucuronide) in this stock solution was determined by direct enzymatic hydrolysis and subsequent colorimetric assay of a portion of the solution according to the analytic procedure.

† The theoretical yield of free pregnanediol from 1 mg. of sodium pregnanediol glucuronide is 0.597 mg.

value obtained by the procedure just described. This finding indicates that the *n*-butanol extraction and partial purification of urinary sodium pregnanediol glucuronide were accomplished with reasonable efficiency. It also suggests that urine extracts prepared according to the analytic procedure are free from substances which inhibit the enzymatic hydrolysis of sodium pregnanediol glucuronide to pregnanediol by the dry, defatted, rat liver powder.

### Comments

The observation that hydrochloric acid hydrolysis tends to damage the water-soluble sodium pregnanediol glucuronide has been reaffirmed.

In an unreported experiment 9.7 mg. of the chemically pure sodium pregnanediol glucuronide (Sample B) were hydrolyzed with acid according to the directions of Astwood and Jones (7). The yield of free pregnanediol was 68 per cent of the theoretical, a value which corresponds closely to the average yield reported by them (average, 68 per cent; maximum, 80 per cent).

Because it has been found previously (10) that pregnanediol can be recovered quantitatively only when it is added to urine as free pregnanediol before simultaneous acid hydrolysis and carbon tetrachloride extraction, it would appear that the acid hydrolysis damages the conjugated sodium pregnanediol glucuronide before hydrolysis to the unconjugated form has rendered it soluble in an organic solvent. Thus, as with sodium dehydroisoandrosterone sulfate (6), it is difficult to imagine steps by which destruction of the conjugated material by acid hydrolysis can be avoided.

On the other hand, the present experience with rat liver enzyme suggests that the enzyme is a reasonably satisfactory agent for the hydrolysis of sodium pregnanediol glucuronide. The recoveries obtained by this enzymatic method of hydrolysis approach 100 per cent sufficiently closely (average 89 per cent) to suggest that the hydrolysis is quantitative and that the losses are due to physical rather than chemical phenomena.

#### SUMMARY

An enzyme which hydrolyzes sodium pregnanediol glucuronide to unconjugated pregnanediol in satisfactory yields has been demonstrated in acetone-dried rat liver powder.

This hydrolysis is carried out by incubating the substrate in aqueous solution at pH 5 for 4 hours at 37°.

Pregnanediol added to urine as sodium pregnanediol glucuronide can be recovered quantitatively as pregnanediol after *n*-butanol extraction of the urine and enzymatic hydrolysis of the extract.

We are indebted to Dr. E. B. Astwood for aid in the preparation of the enzyme and to Dr. A. Stanley Cook of Ayerst, McKenna and Harrison, Ltd., for generous supplies of crystalline sodium pregnanediol glucuronide.

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# THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS

## I. VALINE, LEUCINE, AND ISOLEUCINE

By K. A. KUIKEN, WILLIAM H. NORMAN, CARL M. LYMAN,  
FRED HALE, AND LOIS BLOTTER

(From the Texas Agricultural Experiment Station, Agricultural and Mechanical  
College of Texas, College Station)

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The present chemical methods for the determination of amino acids are in many instances laborious and time-consuming, and in some cases do not give results of unquestioned accuracy. Better analytical methods are particularly needed for those amino acids the chemical structures of which are so similar that quantitative separations by chemical and physical means are almost impossible. Since slight changes in chemical structure often result in pronounced differences in biological response, microbiological methods such as those which have proved useful and dependable in vitamin assays appear to offer definite possibilities for the determination of amino acids in the proteins of foodstuffs.

A method for the determination of valine, leucine, and isoleucine in which *Lactobacillus arabinosus* 17-5 is used as the test organism<sup>1</sup> is described in the present communication. The general procedure of using the organism for biological assay is similar to that described by Snell and Wright (1) for the determination of nicotinic acid. A suitable mixture of pure amino acids is used to replace the casein hydrolysate in the medium for nicotinic acid assay. *p*-Aminobenzoic acid and also a concentrate prepared from tomato juice are added.

The tomato concentrate was first prepared to supply a deficiency in the medium that can now be attributed to *p*-aminobenzoic acid. The essential nature of *p*-aminobenzoic acid for the growth of *Lactobacillus arabinosus* was discovered by Isbell (2). Although the tomato eluate is a rich source of *p*-aminobenzoic acid, its activity is not completely replaced by this vitamin. Evidence has been obtained to indicate that tomato juice contains an unknown growth-stimulating substance for *Lactobacillus arabinosus*.

During the preparation of this manuscript, Brand, Ryan, and Regnery (3) reported a method for determining leucine in which a mutant of the mold *Neurospora crassa* was used as the test organism.

<sup>1</sup> Cultures of this organism may be secured from the American Type Culture Collection, Georgetown University Medical School, Washington, D. C., where it is listed as No. 8014.

## EXPERIMENTAL

*Materials and Methods*

**Basal Medium**—The composition of the complete medium for *Lactobacillus arabinosus* is given in Table I. Assay media for valine, for leucine, or for isoleucine are prepared by omitting the appropriate amino acid.

The medium is prepared from stock solutions which are stored in the refrigerator. Tyrosine is dissolved in 0.1 N NaOH; cystine is dissolved in 0.1 N HCl; all other amino acids are dissolved in water and adjusted to neutrality with a water solution of phenol red as an indicator. Small amounts of this indicator do not appear to affect the growth of the bacteria.

TABLE I  
Complete Medium for *Lactobacillus arabinosus*

Glucose	40 gm.	<i>l</i> (+)-Arginine hydrochloride	400 mg.
Sodium acetate (anhydrous)..	14.5 "	<i>dl</i> -Alanine	400 "
Adenine sulfate	10 mg.	<i>dl</i> -Aspartic acid	800 "
Guanine hydrochloride	10 "	<i>dl</i> -Glutamic acid monohydrate	800 "
Uracil	10 "	<i>l</i> (-)-Histidine monohydrochloride	400 "
Thiamine chloride	200 γ		
Pyridoxine hydrochloride	200 "	<i>l</i> (+)-Lysine hydrochloride	400 "
Calcium pantothenate	200 "	<i>dl</i> -Phenylalanine	400 "
Biotin	0.8 "	<i>l</i> (-)-Proline	400 "
Riboflavin	400 "	<i>dl</i> -Serine	400 "
Nicotinic acid	800 "	<i>l</i> (-)-Tryptophane	400 "
<i>p</i> -Aminobenzoic acid	10 "	<i>dl</i> -Methionine	400 "
Tomato eluate	200 mg.	<i>dl</i> -Threonine	400 "
K <sub>2</sub> HPO <sub>4</sub>	1 gm.	<i>dl</i> -Tyrosine	400 "
KH <sub>2</sub> PO <sub>4</sub>	1 "	<i>dl</i> -Valine	400 "
MgSO <sub>4</sub> ·7H <sub>2</sub> O	400 mg.	<i>dl</i> -Leucine	400 "
NaCl	20 "	<i>dl</i> -Isoleucine	400 "
FeSO <sub>4</sub> ·7H <sub>2</sub> O	20 "	<i>l</i> (-)-Cystine	400 "
MnSO <sub>4</sub> ·4H <sub>2</sub> O	20 "		

Adjust to pH 6.5 to 6.8 and dilute with water to 1 liter

A concentration of 5 mg. per ml. is used for cystine, 20 mg. per ml. for glutamic and aspartic acids, and 10 mg. per ml. for all other amino acids. Synthetic amino acids in so far as they are available are used in preference to amino acids isolated from natural materials. Directions for the preparation of stock solutions of other ingredients of the medium are given by Snell and Wright (1). Preservatives such as toluene or chloroform are not used.

**Tomato Eluate**—The contents of a large can of tomato juice (1350 ml.) are diluted with an equal volume of distilled water and centrifuged. The supernatant fluid is clarified by mixing with 120 gm. of Filter-Cel<sup>2</sup> and

<sup>2</sup> Johns-Manville Hyflo Super-Cel.

filtering with suction. The clear filtrate is adjusted to pH 3 with sulfuric acid. 40 gm. of norit A are added and the suspension is shaken mechanically for 30 minutes. The norit A, on which the active material is adsorbed, is collected by filtering through a thin mat of Filter-Cel. The norit is suspended in 250 ml. of 50 per cent ethanol and then collected by filtering through the original filter mat. The active material is next eluted from the norit as follows: The norit and the filter mat are suspended in 200 ml. of a pyridine-ethanol-water mixture (1:2:1 by volume). The suspension is heated to approximately 60° and shaken mechanically for 15 minutes. The eluate is collected by filtering through Filter-Cel and the norit is treated with the pyridine-ethanol-water mixture in the same manner two additional times. The filtrates are combined and evaporated nearly to dryness by vacuum distillation. A small amount of distilled water is added and the solution is neutralized with sodium hydroxide and the vacuum distillation is then continued until the pyridine is completely removed.

Further purification and concentration of the active material are accomplished by hydrolyzing with sulfuric acid in order to break down protein impurities and then repeating the adsorption and elution processes. The material is refluxed for 24 hours with 40 ml. of 8 N  $\text{H}_2\text{SO}_4$ . Then a hot saturated solution of barium hydroxide is added until the hydrolysate is alkaline to Congo red but acid to litmus. The  $\text{BaSO}_4$  is removed and washed twice with hot water. The hydrolysate and washings are combined and adjusted to pH 3. Any precipitate which forms at this point is removed by centrifuging. The adsorption and elution are then repeated as described above with 8 gm. of norit A and 100 ml. portions of the elution mixture. After removal of the pyridine-ethanol-water mixture by vacuum distillation, a water solution containing 5 mg. of dry solids per ml. is prepared. During the elution some of the norit may become colloidal and hence extremely difficult to filter. This difficulty can be overcome by adding a little fresh norit.

*Assay Procedure*—Stock cultures of *Lactobacillus arabinosus* are carried as stabs in a solid medium containing agar (0.8 per cent), peptonized milk (1 per cent), tryptone (1 per cent), and filtered tomato juice (200 ml. per liter). Four stabs are prepared at monthly intervals from previous stab cultures. They are incubated for 24 hours at 35° and then stored in the refrigerator until needed. Cultures for the inoculum are prepared by transferring material from one of these stabs to tubes containing the above medium with the agar omitted. These cultures are incubated 18 to 24 hours at 35°. The cells are then centrifuged out aseptically and washed once with 0.9 per cent NaCl solution. The washed cells are again suspended in the salt solution and 1 drop of this suspension is added to each assay tube. Best results are obtained with very dilute cell suspensions. The authors have found the use of the peptone-tryptone-tomato juice medium



more desirable for the preparation of the inoculum than the medium described in Table I. When the recommended medium is used, it is entirely satisfactory to prepare the inoculum for tests on successive days by serial transfer from that of the preceding day.. However, it is advisable to return to the original stab cultures weekly to reduce the chance for contamination.

The assay is carried out as follows: Graded amounts of the pure amino acid solution are added in duplicate to a series of tubes which are to be used as standards. The range of the standards is from 0 to 0.1 mg. of *dl*-valine, *dl*-leucine, or *dl*-isoleucine; unexplainable irregularities have occurred when the range was extended beyond these limits. Successive tubes in the series should not differ by more than 0.02 mg. of the amino acid in question. Aliquots of protein hydrolysates or other unknowns prepared as described below (adjusted to pH 6.5 to 6.8) are added to other tubes in duplicate at three levels. 5 ml. of the appropriate assay medium are then added to all tubes and this is followed by the addition of distilled water to adjust the final volume of each tube to 10 ml. The contents of the tubes are mixed by shaking before they are plugged with cotton and sterilized by autoclaving at 15 pounds pressure for 15 minutes. If the contents of the tubes are not thoroughly mixed before being autoclaved, wide variations often occur in the titration values for duplicate tubes. It is also desirable to autoclave the unknown and standards simultaneously. The sterilized tubes are cooled, inoculated, and incubated at 35° for 72 hours.

After incubation the tubes are centrifuged and 5 ml. aliquots are titrated with 0.1 N NaOH, brom-thymol blue being used as the indicator. The clear solutions obtained by centrifuging out the bacteria can be titrated more accurately than the turbid cultures.

*Standard Curves*—Typical standard curves are shown in Figs. 1, 2, and 3.

*Preparation of Hydrolysates for Analysis*—Purified proteins are hydrolyzed by refluxing for 24 hours with 5 N H<sub>2</sub>SO<sub>4</sub>. Approximately 40 ml. of acid are used per gm. of protein. The hydrolysates are neutralized with a hot saturated solution of barium hydroxide. The BaSO<sub>4</sub> precipitate is removed and extracted three times with boiling water. All filtrates are combined and tested for the absence of barium ions, adjusted to pH 6.5 to 6.8, and then diluted to a suitable volume. Natural foodstuffs are first dried *in vacuo* for 5 hours at 95°, and then extracted with dry ethyl ether for 16 hours. The fat-free samples are hydrolyzed in the manner described. No attempt is made to remove carbohydrates.

*Optical Isomers of Amino Acids*—In this paper the naturally occurring amino acids are designated as the *l* forms, the direction of rotation of the free amino acid in water at 25° being given in parentheses. *l*(+)-Valine was resolved from the synthetic *dl* mixture by the method of Holmes and Adams (4). *d*(-)-Valine was obtained from Hoffmann-La Roche and

Company. Synthetic *dl*-isoleucine was repeatedly recrystallized from 80 per cent ethanol for the purpose of removing any alloisoleucine which might be present (see Abderhalden and Zeisset (5)).<sup>a</sup> *d*(-)-Isoleucine was

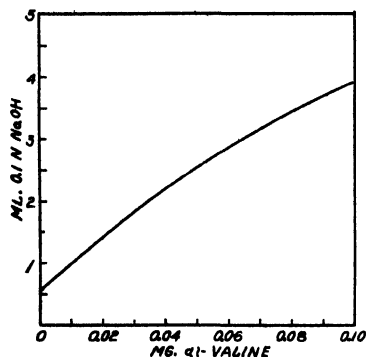


FIG. 1

FIG. 1. Standard curve for the determination of valine. Titration values are for 5 ml. aliquots from 10 ml. culture tubes.

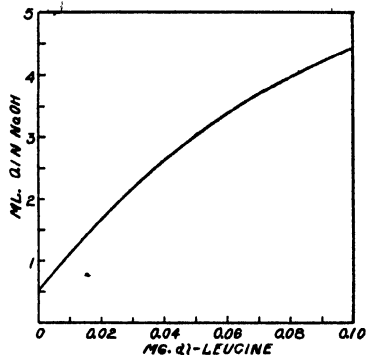


FIG. 2

FIG. 2. Standard curve for the determination of leucine. Titration values are for 5 ml. aliquots from 10 ml. culture tubes.

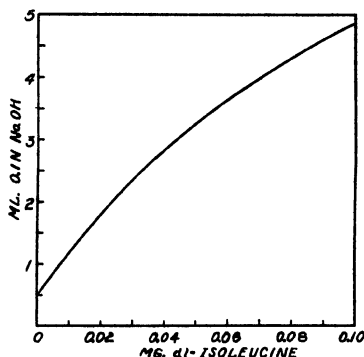


FIG. 3. Standard curve for the determination of isoleucine. Titration values are for 5 ml. aliquots from 10 ml. culture tubes.

resolved from the *dl* mixture by the method of Locquin (6) and *l*(+)-isoleucine by a modification of the method of Holmes and Adams (4). *l*(-)-Leucine was obtained from Amino Acid Manufactures, University of California.

**Preparation of Peptonized Casein**—100 mg. of casein were heated under a reflux condenser for 4 hours with 10 ml. of *N* HCl. The hydrolysate was neutralized with NaOH and diluted to a suitable volume.

*Hydrolysis of Dipeptides*—71.8 mg. of glycyl-*l*(-)-leucine or *dl*-leucylglycine (equivalent to 50 mg. of leucine) were heated in a boiling water bath with 5 ml. of 5 N sulfuric acid for 24 hours. The hydrolysates were neutralized with NaOH and diluted to a suitable volume.

*Amino Acid Requirements of Lactobacillus arabinosus*

As is shown in Table II, nine amino acids were found to be essential for the growth of *Lactobacillus arabinosus*. In the absence of any one of these either no growth or only very scant growth occurred. In addition each of the amino acids listed as accessory increased the growth of the bacteria and maximum growth was obtained only when all of these were present in the medium. A list of amino acids which were found to have no effect on the growth of this organism is also included in Table II.

TABLE II  
*Amino Acid Requirements of Lactobacillus arabinosus*

Essential	Accessory	Amino acids with no effect on growth of <i>Lactobacillus arabinosus</i>
Glutamic acid	Alanine	Norvaline
Tryptophane	Arginine*	Norleucine
Threonine	Aspartic acid	$\alpha$ -Aminoisobutyric acid
Valine	Histidine	$\alpha$ -Amino- <i>n</i> -butyric "
Leucine	Proline	Glycine
Isoleucine	Serine	Hydroxyproline
Cystine	Methionine	$\beta$ -Hydroxyglutamic acid
Lysine	Tyrosine	$\beta$ -Alanine
Phenylalanine		

\* Arginine can be replaced by either ornithine or citrulline.

On occasion even highly purified samples of amino acids have been found to contain traces of other amino acids. Because of this, it is possible that some of the amino acids classed here as accessory may actually be essential, failure to demonstrate their essential nature being a result of their presence as impurities in other amino acids used in the medium.

*Optical Specificity*—Pure samples of valine, leucine, and isoleucine are more readily available as the synthetic *dl* forms. In order that these *dl* preparations might be used as standards, the ratio of their activity to the activity of the naturally occurring *l* forms was determined. The data in Table III indicate that only the naturally occurring optical isomers of these three amino acids are active. Therefore, two weight units of a *dl* form are exactly equivalent in activity to one weight unit of the pure *l*-amino acid.

*Growth Stimulation by Tomato Eluate*

The complete medium as described above contains optimum quantities of the amino acids which affect the growth of *Lactobacillus arabinosus*. In-

creasing the amounts of any of these or of any of the vitamins contained in the medium did not result in increased growth of the organism. Increased growth was obtained, however, by the addition of the tomato eluate preparation as shown by the data for a typical test given in Table IV. The following compounds were found to be completely inactive in increasing growth on the complete medium without added tomato eluate: inositol, pimelic acid, glucosamine, glutamine, asparagine, ascorbic acid, and creatinine.

TABLE III

*Specificity of Lactobacillus arabinosus for Optical Isomers of Valine, Leucine, and Isoleucine*

Amino acid	Optical form	Weight per test	Titration values, * 0.1 N NaOH	
		mg.	ml.	ml.
Valine .		0.00	0.56	0.54
"	d(-)	0.02	0.52	0.54
"	l(+)	0.02	2.17	2.19
"	dl	0.04	2.19	2.19
Isoleucine		0.00	0.21	0.22
"	d(-)	0.04	0.22	0.24
"	l(+)	0.04	2.83	2.84
"	dl	0.08	2.75	2.76
Leucine .	l(-)	0.04	2.70	2.80
"	dl	0.08	2.85	2.82

\* 5 ml. aliquots from 10 ml. culture tubes.

TABLE IV

*Growth-Stimulating Effect of Tomato Eluate*

The medium was complete except for the omission of tomato eluate.

Eluate added per tube	Titration values, * 0.1 N NaOH
mg	ml
0.0	4.00
0.5	5.46
1.0	6.33
2.0	6.50

\* 5 ml. aliquots from 10 ml. culture tubes.

*Effect of Humin Formation on Determination of Valine, Leucine, and Isoleucine*

It is well known that the presence of carbohydrates during the hydrolysis of proteins in acid solution results in increased formation of humin with corresponding loss of protein nitrogen. The loss of tyrosine and tryptophane in this manner is particularly significant. An attempt has been

made to evaluate the loss of valine, leucine, and isoleucine resulting from humin formation when hydrolysis is carried out in the presence of carbohydrates. Casein was analyzed for valine, leucine, and isoleucine. The analyses were then repeated on samples of casein which had been hydrolyzed in the presence of different carbohydrates. The data presented in Table V indicate, as was expected, that some loss of these amino acids does occur as a result of humin formation. However, the data would seem to indicate

TABLE V

*Effect of Hydrolyzing Casein in Presence of Carbohydrates on Determination of Valine, Leucine, and Isoleucine*

Average values for three different test levels.

Carbohydrate added to 1 gm casein before hydrolysis	Valine	Leucine	Isoleucine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
None . . . . .	6.25	9.27	6.05
Sucrose, 1 gm. . . . .	6.20	8.98	5.97
Arabinose, 1 gm. . . . .	5.83	9.02	5.55
Soluble starch, Merck, 5 gm. . . . .	6.00	9.07	6.10

TABLE VI

*Valine, Leucine, and Isoleucine Content of Some Foodstuffs*

Material analyzed	Protein* content of un-dried samples	Valine			Leucine			Isoleucine		
		Un-dried basis	Moisture-free basis	Per cent total N as valine	Un-dried basis	Moisture-free basis	Per cent total N as leucine	Un-dried basis	Moisture-free basis	Per cent total N as isoleucine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Cottonseed meal	42.75	1.59	1.70	2.78	2.13	2.28	3.32	1.45	1.55	2.27
Peanut meal	44.69	1.79	1.95	3.00	2.45	2.67	3.66	1.53	1.67	2.28
Soy bean meal	43.91	1.85	2.06	3.15	2.90	3.23	4.40	2.06	2.29	3.14
Wheat	13.01	0.51	0.58	2.66	0.82	0.93	3.84	0.47	0.53	2.20
Gelatin			2.46			3.30			1.71	

\* Kjeldahl N multiplied by the factor 6.25 except for wheat, for which the factor 5.7 was used.

that this loss is not great enough to affect seriously the usefulness of determinations of these amino acids in the proteins of natural foodstuffs.

#### *Analytical Data for Foodstuffs*

Table VI gives data for the valine, leucine, and isoleucine yielded by the proteins of a few natural foodstuffs.

*Effect of Omitting Tomato Eluate from Assay Medium*—Simplification of the medium by omitting the tomato eluate would be an advantage if this

could be done without sacrificing the accuracy of the determinations. This would appear to be possible if the material to be analyzed does not contain a significant amount of the unknown growth-stimulating substance. In cases in which the food material does contain the growth-stimulating substance, high results are to be expected if the eluate is omitted from the medium. The data in Table VII were obtained by analyzing a few foodstuffs, media with and without the tomato eluate being used. Although these data would indicate that in some cases satisfactory results can be obtained by either procedure, it has been found advisable to include the tomato eluate in the assay medium in all cases, because better agreement between titrations of duplicate tubes and smoother standard curves have been consistently obtained when it is used.

TABLE VII

*Effect of Omitting Tomato Eluate from Assay Medium on Determination of Leucine*

Material analyzed	Leucine content, undried basis	
	Without tomato eluate in medium	With tomato eluate in medium
	<i>per cent</i>	<i>per cent</i>
Cottonseed meal	2.07	2.03
Peanut meal	2.48	2.45
Soy bean meal	2.93	2.93
Wheat ..	0.81	0.76

#### *Effect of Incomplete Hydrolysis*

The possibility has been considered that amino acids might be determined in partially hydrolyzed materials by the use of the microbiological method. The drastic conditions usually employed for hydrolysis would be avoided by this procedure. Casein, peptonized by being heated for 4 hours with 1 N hydrochloric acid, was assayed for valine, leucine, and isoleucine, and the values were compared with those obtained after complete hydrolysis. Decidedly low results were obtained with the peptonized samples (Table VIII).

This effect was also studied with two dipeptides containing leucine which were assayed both before and after hydrolysis with sulfuric acid. Satisfactory recoveries of leucine were obtained with the hydrolyzed samples of both dipeptides and also with the unhydrolyzed glycylleucine at the lower test levels (Table IX). Unsatisfactory recoveries were obtained with unhydrolyzed leucylglycine and also with unhydrolyzed glycylleucine when tested at the higher level. It appears that the extent to which an amino acid in a peptide is utilized by this organism depends on the test level and

also on the nature of the peptide. The results obtained with the partially hydrolyzed casein and the unhydrolyzed peptides indicate that samples should be completely hydrolyzed before analysis.

TABLE VIII

*Effect of Incomplete Hydrolysis on Analysis of Casein for Leucine, Isoleucine, and Valine*

Treatment of casein sample	Valine	Leucine	Isoleucine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Complete hydrolysis	6.25	9.27	6.05
Incomplete hydrolysis (peptonization)	5.45	8.35	4.91

TABLE IX

*Effect of Hydrolysis on Determination of Leucine in Peptides*

	Theoretical l(-)-leucine content of samples	l(-)-Leucine found			
		Non-hydrolyzed sample		Hydrolyzed sample	
		<i>mg.</i>	<i>per cent of theoretical</i>	<i>mg</i>	<i>per cent of theoretical</i>
<i>dl</i> -Leucylglycine	0.02	0.0167	84	0.0205	102
	0.04	0.0323	81	0.0400	100
	0.08	0.0610	76	0.0820	102
Glycyl-l(-)-leu- cine	0.02	0.0195	98	0.0195	98
	0.04	0.0380	95	0.0415	104
	0.08	0.0638	80	0.080	100

## DISCUSSION

The method described is directly applicable to the determination of valine, leucine, and isoleucine in foodstuffs as well as in purified proteins. The loss of these three amino acids because of the humin formation which takes place when foodstuffs are hydrolyzed in acid solution would appear to be relatively small even though the carbohydrates are not removed. Hydrolysis by the use of strong alkali is not a satisfactory procedure for the preparation of samples, because alkaline hydrolysis brings about racemization and only the naturally occurring forms of these amino acids are utilized by *Lactobacillus arabinosus*. For the same reason, if small amounts of the *d* or "unnaturally occurring" forms of valine, leucine, or isoleucine are present in foodstuffs or are formed during acid hydrolysis, these will not be determined by the method as described in this paper. Details of a method by which foodstuffs may be hydrolyzed in acid solution without the formation of humin are being studied and will be reported in a later communication.

The extreme sensitivity of the method makes possible the detection of small amounts of valine, leucine, or isoleucine in other amino acid preparations. For example, 0.5 per cent or even less of leucine present as an impurity in an isoleucine preparation can be detected and quantitatively measured. This sensitivity offers the further advantage of permitting analyses to be carried out on relatively small samples. Although the data reported in this paper were obtained with samples selected to contain about 1 gm. of protein, much smaller samples may be used if desired.

Potentially the method as described in this paper might be used for the determination of any of the amino acids which are essential for the growth of *Lactobacillus arabinosus*. Specificity tests for the purpose of establishing the reliability of the assays will be particularly necessary in the case of some of these amino acids. For example, Snell (7) has shown that anthranilic acid and also indole can partially replace tryptophane for the growth of this organism. These substances if present in the samples to be analyzed would interfere with the determination of tryptophane. A second problem in this regard is that the tomato eluate as now used contains some amino acids although it is essentially free of valine, leucine, and isoleucine.

The experimental evidence thus far obtained does not justify postulates concerning the chemical nature of the tomato eluate factor. The adsorption and elution processes are very similar to those previously used by Snell and Peterson (8) for the concentration from liver of an eluate factor which is required by *Lactobacillus casei*  $\epsilon$ . However, the tomato eluate factor appears to be different from that described by Snell and Peterson on the basis of the following considerations. The tomato eluate factor is stable to treatment with strong acids, while the liver eluate factor is labile to hydrochloric acid (8) and sulfuric acid (9). Secondly, Hutchings, Bohonos, and Peterson (9) have reported that the liver eluate factor did not stimulate the growth of *Lactobacillus arabinosus*.

#### SUMMARY

1. A microbiological method for the quantitative determination of valine, leucine, and isoleucine in foodstuffs is described. *Lactobacillus arabinosus* 17-5 is used as the test organism.
2. The amino acid requirements of *Lactobacillus arabinosus* are given.
3. Evidence is presented which indicates that tomato juice contains an unknown growth-stimulating factor for *Lactobacillus arabinosus*. The preparation of a concentrate of this factor is described.

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# THE SEDIMENTATION RATE OF THE INFECTIOUS PRINCIPLE OF TOBACCO MOSAIC VIRUS\*

By MAX A. LAUFFER<sup>1</sup>

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton)*

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An important question concerning the macromolecular nucleoprotein isolated from mosaic-diseased tobacco plants (23) is whether this material actually is the infectious principle of tobacco mosaic virus or merely some substance associated with it. Considerable evidence relative to this issue has been amassed. The virus protein is always found associated with the infectious principle; it has been isolated from many different batches of diseased tobacco plants and from other plant species infected with the mosaic disease (4, 16, 26). Similar, but distinctive, proteins have been obtained from plants diseased with strains of tobacco mosaic virus (2, 10, 11, 24). Entirely different proteins have been isolated from tobacco and other plants infected with unrelated diseases (3, 20, 28). The ultraviolet absorption spectrum of the tobacco mosaic virus protein coincides with the radiation range lethal for infectivity (6, 15). The pH stability range of the protein coincides with the stability range of virus infectivity (22, 32). Chemical changes in the protein molecule brought about by prolonged action of formaldehyde (21), of iodine and iodoacetamide (1), of ketene, phenyl isocyanate, carbobenzoxy chloride, *p*-chlorobenzoyl chloride, and benzenesulfonyl chloride (17) result in modification or destruction of virus infectivity. In the case of formaldehyde, reversal of the reaction is accompanied by partial reactivation of the virus. Attempts to separate the infectious principle from the virus protein by centrifugation under a variety of conditions (25) and by fractional crystallization (16) have consistently failed. These facts form a reasonable basis for the assumption that the infectious principle and the virus nucleoprotein are not separable and that virus infectivity is a specific property of the protein. Nevertheless, much of the evidence on which this assumption is founded is essentially negative in character—the inability to demonstrate a difference between the infectious entity and the protein unit.

Further progress in this field can be made by applying more and more sensitive tests for differences. One type of such test can be made with the ultracentrifuge. The size and shape (12; 14, 27) and the rate of sedimenta-

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tion (13, 31) of the predominating nucleoprotein particles in a preparation of tobacco mosaic virus are well known, and it is also known in a general sort of way from the sedimentation results obtained in high speed angle centrifuges (33) and from the ultracentrifugation studies of Bechhold and Schlesinger (5) that the sedimentation rate of the infectious principle is at least of the same order of magnitude. However, much more valuable information can be obtained by comparing the sedimentation rates of the protein and of the infectious principle on the same virus sample. It is the purpose of the present report to describe such experiments and to evaluate the precision of the results.

### *Materials and Methods*

A special type of ultracentrifuge cell, known as a separation cell, was used in this study. This cell differs from the conventional type by having a perforated barrier in the sedimentation column about two-thirds of the distance from the top to the bottom. Its advantage is that it permits the material to be observed by the usual optical methods during the course of the run and also allows a reasonably accurate sample to be removed at the completion of the run for analysis by chemical or biological means. The separation cell was designed by Tiselius, Pedersen, and Svedberg (30). It has been used in previous studies to show that Type I antipneumococcus activity is associated with the fastest globulin component of horse serum (30), that the infectivity of mouse encephalitis virus is probably associated with a component with a sedimentation constant of about 160 Svedberg units (9), and that the oxytocic and pressor activities of ox pituitary are probably associated with a pure protein with a molecular weight of about 30,000 (7).

Virus infectivity measurements were carried out by a method similar to that described by Price and Spencer (18). Standard and unknown virus solutions each at concentrations of about  $10^{-5}$  and  $10^{-6}$  gm. per cc. were applied to opposite halves of *Nicotiana glutinosa* L. leaves. Twelve plants containing six leaves each were used. A type of Latin-square arrangement was worked out in such a manner that all four solutions were applied to every plant and all comparable positions on the plants were occupied the same number of times by each solution. The determination of the relative activities of the control and unknown and the computation of the experimental error by the analysis of variance were carried out in a manner comparable to that described by Price and Spencer for the case of two dilutions.

Two preparations of tobacco mosaic virus, designated as Preparations A and B, were used in these studies. Each was isolated from freshly harvested, young, diseased Turkish tobacco plants by two high and low speed centrifugation cycles.

In the experiments in the separation cell, the cell was filled with solutions containing about 5 mg. of virus per cc. of 0.1 M phosphate buffer at pH 7. It was then spun in the ultracentrifuge until the protein boundary was either just above or just below the perforated barrier. The boundary was followed in each case during the course of the experiment by the cylindrical lens-schlieren method (29). After the completion of each run, the contents of the top section of the separation cell were removed quantitatively. From the optical data on the sedimenting boundary, the sedimentation constant of the nucleoprotein was calculated in the usual manner. Also from the data, the position of the boundary at the end of each run was determined. From the position of the boundary with respect to that of the barrier at the end of an experiment, the fraction of the original protein remaining in the top compartment was calculated in the cases of the experiments in which the boundary did not migrate beyond the barrier. This computation is dependent upon the assumption that the protein is practically homogeneous with respect to sedimentation rate. Analyses for protein by a micro-Kjeldahl method and for virus infectivity were made upon the original virus solutions as introduced into the cell and upon the contents removed from the top compartment.

#### EXPERIMENTAL

In Experiment I, virus Preparation A was centrifuged until the protein boundary had migrated about three-fourths of the way to the barrier. The sedimentation constant for the 5 mg. per cc. solution, corrected to water at 20°,  $s_{20}^0$ , was calculated from the optical data to be 165 Svedberg units.<sup>1</sup> This would correspond to a value at infinite dilution of about 187 Svedberg units (13).<sup>2</sup> It can be seen in Table I that the fraction of the protein remaining in the top compartment after the centrifuge was stopped, as calculated from the position of the boundary at the end of the experiment, agreed excellently with that calculated from chemical analyses. This is evidence that virus Preparation A really was quite homogeneous and also that the sampling process was fairly accurate. It may also be seen that the fraction of the virus infectivity remaining in the upper compartment differed from the fraction of protein by an amount just about equal to the probable error of the infectivity measurement. This result means that the infectious principle must sediment at a rate not demonstrably different from that of the protein particles.

In order to appreciate the full significance of this result, it is necessary to look at it from a slightly different point of view. From the value of 0.21 for the ratio of activity of the top compartment to original activity, one can

<sup>1</sup> The Svedberg unit is  $10^{-13}$  cm. per second per unit field.

<sup>2</sup> Lauffer, M. A., unpublished results.

calculate a sedimentation constant of 178 Svedberg units for the infectious principle. This may be compared with the value 165 calculated from the optical data for the protein in this same experiment. From the upper and lower limits of the probable error of the ratio of final to initial activities, one can calculate lower and upper limits of the probable error of the sedimentation constant of the infectious principle to be 167 and 188 Svedberg units; that is, 178 Svedberg units  $\pm$  6 per cent. The lower value is seen to be in excellent agreement with the sedimentation constant of the virus protein. Thus, the sedimentation rate of the infectious principle agrees with that of the protein within a probable error of 6 per cent.

TABLE I  
*Sedimentation of Tobacco Mosaic Virus in Separation Cell*

Material	Fraction above barrier after run
Protein determined optically. ....	0.26
" " by chemical analysis. ...	0.26
Virus infectivity . . . . .	0.21 $\pm$ 0.045*

\* The number 0.045 is the probable error. The expression  $0.21 \pm 0.045$  means that the chances are even that the true infectivity ratio lies within the range 0.165 to 0.255. The probable error was calculated from the standard error, which was obtained from the infectivity data in a manner almost exactly parallel to that outlined in detail by Price and Spencer (18) in their discussion, under Scheme I, of the data of their Table I. In this case, however, the compositions of the variations between leaves and within leaves were slightly different. The variation between leaves was considered to be made up of four parts, namely, that contributed by the different plants, that contributed by the differences in leaf position, that contributed by the difference between right and left halves at two concentrations (this term includes the  $B^2$  of Price and Spencer), and the residual error. The variation within leaves was considered in the present case to be made up of only the quantity designated by Price and Spencer as  $D^2$  and the residual error.

This result is entirely consistent with the assumption that the infectious principle and the protein are identical, but it does not rule out the possibility that they differ slightly with respect to sedimentation rate. It is, however, possible to carry out experiments which will define the maximum possible difference between these two sedimentation rates. If the protein is sedimented until the boundary goes a small distance beyond the barrier, and if virus infectivity measurements on the contents of the top compartment then show only a trace of infectivity, it is possible to conclude with practical certainty that the sedimentation rate of the infectious principle is not very much less than that of the protein. Experiment II was carried out in just such a manner, with virus Preparation A. The protein boundary was allowed to move 13.4 per cent beyond the barrier. Then the top compart-

ment was found to have retained only about 0.1 per cent of its original infectivity. As will be shown later, this small amount of residual infectivity is probably due to contamination. Thus, it may be concluded with safety that the sedimentation rate of the infectious principle could not have been less than 88 per cent of that of the protein.

In order to obtain a value for  $s_{20}^0$  which could not possibly be exceeded by the true sedimentation rate of the infectious principle, Experiment III was carried out on virus Preparation B. The boundary was allowed to migrate all but 15 per cent of the way to the barrier. Protein analyses and infectivity analyses, respectively, showed that the material in the top compartment contained 11 and 5 per cent of the original. These values do not agree as well as those of Experiment I, possibly owing either to less quantitative recovery of the components of the top compartment or to slight inhomogeneity of virus Preparation B. Nevertheless, it is perfectly clear that, if the infectious principle had a sedimentation constant equal to or more than 100/85 times that of the protein, all but a trace of the infectivity would have been behind the barrier. Hence, this experiment shows that the sedimentation constant of the infectious principle is almost certainly not more than 17 per cent greater than that of the protein. The last two experiments show that the sedimentation constant of the infectious principle could not be less than 88 per cent nor more than 117 per cent of that of the protein.

Experiment IV was carried out to determine whether the small amount of infectivity remaining in the top compartment in Experiment II was due to the association of a small amount of infectivity with small particles or simply to contamination. Virus Preparation B was centrifuged at a concentration of about 10 mg. per cc. in the separation cell until the protein boundary had migrated about 10 per cent beyond the barrier. The content of the top compartment was removed and tested for infectivity relative to the original. It was then diluted 1:3 with 0.1 M  $\text{PO}_4$  and subjected to an exactly comparable run in the separation cell. The loss of infectivity of the content of the top compartment in this second run was also measured. The fractions of infectivity removed from the top compartment were about the same for the two runs—about 99 per cent. This result demonstrates that the small amount of infectivity remaining above the barrier when the principal protein component is sedimented beyond the barrier may be due largely to unavoidable contamination and is probably not due to the association of a small amount of infectivity with smaller particles.

#### DISCUSSION

The fact that the sedimentation constant of the infectious principle agrees within a probable error of  $\pm 6$  per cent with that of the protein and could not possibly be less than 88 per cent nor more than 117 per cent of

that of the protein is excellent quantitative evidence in support of the assumption that the predominating protein particles in a tobacco mosaic virus preparation are actually the bearers of the infectivity. This result is perhaps the most precise quantitative correlation of the infectious principle with the virus protein yet obtained. Nevertheless, these data do not eliminate entirely the possibility that the infectious principle is associated with particles slightly different from the predominating protein particles but present at too small a concentration to be detected by other than biological means. They do narrow greatly the possibilities for such particles, however. For example, it has been shown that rod-like particles having the same diameter but one-half the length of the predominating protein particles should have a sedimentation constant of about 145 Svedberg units (12, 14). The data of this study indicate that it is just barely within the realm of possibility that all of the infectivity of tobacco mosaic virus is carried by such half units of the predominating protein particles, but that this situation is very unlikely due to the small probable error of the sedimentation rate of the infectious principle. The present data exclude entirely the possibility that the infectious principle is borne solely by fragments of the predominating protein particles smaller than half size. It has also been shown that particles obtained by the end to end aggregation of two of the predominating virus protein particles should sediment with a rate of 204 Svedberg units (12, 14). The present data exclude entirely the possibility that virus infectivity is carried solely by such dimers of the predominating particles.

The question as to whether any infectivity may be associated with fairly small particles requires further consideration. If all of the residual infectivity in the upper compartment after the first centrifugation in Experiment IV had been due to small particles with sedimentation rates of the order of 100 Svedberg units, it can be calculated that from 3 to 4 per cent of the infectious particles of virus Preparation B must have belonged to the smaller group, since from one-third to one-fourth of such particles would have remained above the barrier when the component with the sedimentation rate of 165 Svedberg units migrated about 10 per cent beyond the barrier, and since about 1 per cent of the infectivity actually remained behind. If this situation had obtained, on the second centrifugation run in Experiment IV about 25 to 30 per cent of the infectivity should have remained above the barrier, for the starting material would have been composed of almost nothing except the small component. Actually, only 1 per cent remained above. Thus, at most only 3 or 4 per cent of the infectivity remaining above the barrier in the first sedimentation run could possibly have been due to small particles. The bulk of it, if not all of it, must have been due to unavoidable contamination. In the original virus Preparation B, therefore,

considerably less than 0.1 per cent of the infectivity could possibly have been due to particles with a sedimentation rate of about 100 Svedberg units. Even if all of the residual infectivity in the top compartment in Experiment II had been due to material with a sedimentation rate of about 100 Svedberg units, only a few tenths of 1 per cent of the infectivity of Preparation A could possibly have been associated with this component. It is unnecessary to assume that any infectivity is associated with such a small component. Particles with a sedimentation rate of 100 Svedberg units would correspond to protein spheres with a radius of about  $11\text{ m}\mu$  or with rods  $15 \times 37\text{ m}\mu$ . Thus, the results of the present investigation are entirely at variance with the basic considerations forming the background of a recently published opinion to the effect that the fundamental infectious unit of tobacco mosaic virus is a particle with dimensions of  $15 \times 37\text{ m}\mu$  (8), and they thereby lend support to Rawlins' criticism of that opinion (19).

The results obtained in the present study support the conclusion that the bulk of the infectious principle of tobacco mosaic virus is firmly associated with the predominating nucleoprotein particles. They eliminate entirely the possibility that the bulk of the infectivity is associated with particles very much smaller or very much larger than the predominating particles. Finally, the present results are incompatible with the assumption that any more than a trace of infectivity is associated with particles small enough to have a sedimentation constant of about 100 Svedberg units, and they are entirely consistent with the assumption that no infectivity is borne by such particles.

#### SUMMARY

The sedimentation constant of the infectious principle of tobacco mosaic virus was determined in the ultracentrifuge with the aid of a separation cell. It was found to be the same as that of the virus protein within a probable error of 6 per cent. It was found further that the maximum possible deviations of the sedimentation constant of the infectious principle from that of the protein were +17 per cent and -12 per cent. This result strengthens greatly the case for believing that tobacco mosaic virus infectivity is a property of the nucleoprotein particles predominating in a virus preparation, and it eliminates entirely the possibility that the sole carriers of virus infectivity are particles any smaller than half the size of the predominating particles.

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# THE CYSTEINE, CYSTINE, AND METHIONINE CONTENT OF PROTEINS

BY W. C. HESS AND M. X. SULLIVAN

(From the Chemo-Medical Research Institute, Georgetown University, Washington)

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In the study of the cystine content of proteins over a period of years (1), there have been only a few instances in which the hydrochloric acid hydrolysates of these proteins gave any evidence for the presence of cysteine, possibly because whatever cysteine complexes there may have been in the protein were oxidized during the preparation of the hydrolysate. The muscle proteins of meat and fish, tobacco mosaic virus protein, and some samples of egg albumin gave indications of the presence of cysteine. There have been many reports on the presence of SH groups attributed to cysteine in unhydrolyzed protein, both before and after denaturation by a wide variety of methods. Numerous methods of determining quantitatively the amount of SH groups present have been suggested. Heffter (2) early showed that egg albumin, which gave no nitroprusside test in its native state, did so after coagulation by heat. Many others have employed the nitroprusside test as a qualitative measure of SH groups. Todrick and Walker (3) heated protein suspensions with varying amounts of 2,6-dichlorophenol indophenol, a dye which reacts slowly with protein SH groups at ordinary temperature. This reaction, however, is not stoichiometric. Mirsky and Anson (4) estimated the protein SH by means of the phosphotungstic acid reagent of Folin and Looney (5) applied to hydrolysates before and after oxidation of the SH groups by potassium ferricyanide or combination with sodium iodoacetate. Kuhn and Desnuelle (6) introduced the use of porphyrindin for the estimation of protein SH groups. This method was used by Greenstein (7-9) in a series of studies of a number of proteins, both in their native state and when denatured with urea or guanidine salts. Hellerman, Chinard, and Ramsdell (10) and Hellerman, Chinard, and Deitz (11) have used the *o*-iodosobenzoate ion to determine the SH groups in egg albumin and urease. These various procedures would give positive results with any compound that contains an SH group, and it remains to be demonstrated that the reactions given by the proteins are actually due to the presence of cysteine complexes.

If the proteins contain both cystine and cysteine, it should be possible to determine both of these amino acids in acid hydrolysates. Sullivan, Hess, and Howard (12) have described a procedure for the separate determination of cystine and cysteine in mixtures of these two amino acids. By the use

of this procedure, the cysteine yielded by the protein can be determined following acid hydrolysis, provided there is no loss of cysteine during the hydrolysis. The loss of cysteine is primarily associated with humin formation. Sullivan and Hess (13) have employed titanous chloride to prevent humin formation in the acid hydrolysis of protein. In the hydrolysis in the presence of titanous chloride all of the cystine present is converted to cysteine and no differentiation between cystine and cysteine originally present in the protein is possible. The hydrolysis of protein with 57 per cent hydriodic acid in the presence of potassium hypophosphite introduced by Baernstein (14), although having the advantage of diminishing humin formation, has a similar disadvantage in that all of the cystine is reduced to cysteine. Brand and Kassell (15) have found that hydrolysis with 20 per cent hydrochloric acid in the presence of urea will reduce humin formation and permit the determination of cysteine in the hydrolysate. By estimation of the SH groups present before hydrolysis, and subsequent determination of cystine and cysteine following hydrolysis, it should be possible to arrive at some understanding of the relationship of protein cysteine to titratable protein SH groups. At the same time methionine may be estimated and the distribution of the total sulfur can be determined.

#### EXPERIMENTAL

*Proteins*—Nine different proteins from a variety of sources were employed. All of the proteins were freshly prepared samples with one exception and all of the analytical values obtained are expressed upon the ash- and moisture-free basis. The values for the tobacco mosaic virus protein are based on the weight of a sample dried in a vacuum desiccator over phosphorus pentoxide.

Crystalline egg albumin was prepared by the method of Kekwick and Cannan (16). The solution of the egg albumin freed from sodium sulfate by dialysis was reduced to a powder by evaporation under reduced pressure at 3° in a desiccator. The residue was not denatured, since it was completely soluble in water. Serum albumin was prepared from beef blood by the method of Kekwick (17). The same method of drying was employed. The serum albumin likewise was not denatured. Globin was prepared from calf blood by the method of Hamsik (18). The dialyzed solution was evaporated to dryness in a vacuum desiccator over sulfuric acid. The glycinin employed was prepared from defatted soy bean meal according to the procedure of Jones and Csonka (19). The fraction precipitated from 10 per cent sodium chloride solution by ammonium sulfate at 55 per cent saturation was employed. This fraction was reprecipitated several times and finally dialyzed free from salt. The precipitated protein was removed by centrifugation and rapidly dried with alcohol and ether free from perox-

ide. The dry protein was soluble in dilute salt solution. Amandin and excelsin were prepared by the method of Svedberg and Sjögren (20) and the edestin was prepared by a similar method. Again all the salt-free dialysates were centrifuged and the proteins rapidly dried with alcohol and ether. They were all soluble in dilute salt solution. Tobacco mosaic virus protein was prepared by the Ross procedure (21) and was the same sample used by Hess, Sullivan, and Palmes (22). Myosin was prepared from rabbit muscle by the procedure of Greenstein and Edsall (23). The myosin could not be dried without denaturation; so, for all the experiments on the native myosin, a potassium chloride solution was used. The concentration of myosin was determined by estimating nitrogen in an aliquot and using 16.6 per cent as the nitrogen content of the myosin. The dried myosin was prepared from this solution according to the procedure of Greenstein and Edsall (23). Squash seed globulin was prepared by the method of Vickery, Smith, Hubbell, and Nolan (24) from *Cucurbita maxima*, variety Golden Delicious.

*Analytical Methods*—The determination of the SH groups present in the unhydrolyzed proteins was made by an iodine titration according to the Okuda method for cysteine. The aqueous or dilute salt solution of the protein was made to 2 per cent hydrochloric acid and 2.5 cc. of 4 per cent hydrochloric acid and 2.5 cc. of 5 per cent aqueous potassium iodide were added. The solution or suspension was cooled to 20° and titrated to a permanent yellow color with M/600 potassium iodate that had been standardized against cysteine hydrochloride similarly treated and the results were calculated as cysteine. It was found that the same value was obtained whether the titration was conducted in the presence of 8 mm guanidine hydrochloride per cc. of solution or in its absence. The sulphydryl groups of proteins can be titrated by iodine whether the protein is in the native or the denatured state. Denatured proteins in general give a nitroprusside test that is a purplish red color on the addition of sodium nitroprusside and ammonium hydroxide. Proteins after short contact with 2 per cent hydrochloric acid at 20° as previously described do not give the nitroprusside reaction, although some change in solubility may occur, a change which may be the initial phase of denaturation. Anson (25) has shown that native egg albumin, which does not give the nitroprusside test, can react with iodine despite the fact that it does not react with ferricyanide or porphyrindin.

Hydrolysis of the proteins was carried out by three different reagents: 20 per cent hydrochloric acid containing 100 mg. of urea per cc., 20 per cent hydrochloric acid, and 6 N sulfuric acid. It has been found that ordinary c.p. 36 per cent hydrochloric acid, after dilution to 20 per cent concentration, frequently contains oxidizing material that renders the acid unfit for use as a hydrolyzing agent. 20 per cent hydrochloric acid prepared by dis-

tillation of the constant boiling acid is more satisfactory. The hydrolysis in each case was conducted in a stream of nitrogen for a period of 6 hours in the first two procedures and for 8 hours in the third. All of the hydrolysates were carefully brought to pH 3.5 by 5 N sodium hydroxide added

TABLE I  
*Cystine, Cystine, and Methionine Content (Per Cent) of Proteins*

Protein	20 per cent HCl + urea		20 per cent HCl		6 N H <sub>2</sub> SO <sub>4</sub>		Direct titration, cystine	20 per cent HCl, methio- nine
	Cys- teine	Cys- tine	Cys- teine	Cys- tine	Cys- teine	Cys- tine		
Egg albumin. . . . .	1.37	1.03	0.97	1.51	1.42	0.92	1.41	5.07
Serum albumin (bovine)	0.30	5.76	0.21	5.92	0.45	5.70	0.32	0.45
Globin (calf) . . . . .	0.12	0.46	0.10	0.47	0.13	0.45	0.06	0.87
Glycinin (soy bean)	0.33	1.16	0.23	1.25	0.33	1.16	0.39	2.59
Edestin (hemp-seed) . .	0.32	0.96	0.24	1.03	0.28	0.99	0.52	2.18
Excelsin (Brazil-nut). . .	0.16	1.25	0.10	1.31	0.14	1.28	0.17	3.13
Amandin (almond) . . .	0.00	1.37	0.00	1.38	0.00	1.32	0.00	0.43
Tobacco mosaic virus.	0.57	0.11	0.21	0.47	0.68	0.00	0.68	
Myosin (rabbit) . . . .	1.07	0.16	0.94	0.26	1.12	0.10	1.25	4.03
“ “ dry . . . . .	0.00	1.22	0.00	1.04	0.22	0.93		3.84
Squash seed globulin . .	0.49	0.82	0.17	1.14	0.35	0.97	0.51	2.39

TABLE II  
*Total Sulfur and Sulfur Distribution in Proteins*

Protein	Total S	Cystine, cysteine, and methionine S	Percentage ratio
	<i>per cent</i>	<i>per cent</i>	
Egg albumin. . . . .	1.75	1.73	99
Serum albumin (bovine)....	1.84	1.71	93
Globin (calf) . . . . .	0.40	0.34	85
Glycinin (soy bean)....	0.96	0.95	99
Edestin (hemp-seed)....	0.99	0.81	82
Excelsin (Brazil-nut) . . . .	1.06	1.04	98
Amandin (almond) . . . . .	0.44	0.46	105
Myosin (rabbit) . . . . .	1.13	1.19	105
“ “ dry . . . . .	1.13	1.15	102
Squash seed globulin . . . .	0.99	0.86	87

dropwise with stirring, made to volume with 0.1 N hydrochloric acid, and filtered. In general there was less humin formation following hydrolysis with 20 per cent hydrochloric acid containing urea than with the 20 per cent hydrochloric acid alone. Cystine and cysteine were determined by the procedure of Sullivan, Hess, and Howard (12). Methionine was deter-

mined by the method of McCarthy and Sullivan (26). Frequently it was found that the use of concentrated sodium hydroxide and subsequent acidification with concentrated hydrochloric acid resulted in the formation of bubbles in the final stage of the reaction that rendered colorimetric estimation somewhat difficult. The use of the following procedure obviated this difficulty to a considerable degree but did not give values differing from those obtained by the original procedure. To 5.0 cc. of the solution to be tested were added 1.0 cc. of 5 N sodium hydroxide, 1.0 cc. of 1.0 per cent aqueous glycine solution, and 0.2 cc. of aqueous 10 per cent sodium nitroprusside solution. The solution was kept at 35° for 10 minutes, and then chilled in an ice bath and 2.5 cc. of 20 per cent hydrochloric acid added. After being shaken for several minutes, the solutions are ready for colorimetric reading.

Total sulfur was determined in the original protein, and, in some cases, in the hydrolysates by the alkaline permanganate fusion of Pollack and Partansky (27).

The values obtained upon the proteins are given in Tables I and II.

#### DISCUSSION

All of the proteins except amandin and the dried, denatured myosin contained cysteine complexes. In every case in which the direct iodine titration of the unhydrolyzed protein indicated the presence of titratable SH groups, the hydrolysate of the same protein had a cysteine content of the same order of magnitude, although generally slightly lower. This slightly lower cysteine value is probably due to oxidation of the cysteine during hydrolysis, an effect more marked when the hydrolysis is conducted with 20 per cent hydrochloric acid alone than when 20 per cent hydrochloric acid in the presence of urea or when 6 N sulfuric acid is employed.

The SH content of some of these proteins, in the denatured state, has been obtained by other investigators. The values for egg albumin, calculated as cysteine, have been tabulated by Greenstein and Jenrette (9) and range from 0.87 to 1.32 per cent. The latter value is that reported by Hellerman *et al.* (10) in 1941, while more recently Hellerman *et al.* (11) have reported a value of 1.28 per cent. These values are to be compared with that of 1.41 per cent cysteine reported here. Anson and Stanley (28) titrated tobacco mosaic virus protein in guanidine hydrochloride solution by several methods and give a value of 0.71 per cent cysteine. The value reported in the present paper is 0.68 per cent.<sup>1</sup> Greenstein (8, 9, 29), using guanidine hydrochloride as the denaturing agent and porphyrindin as the

<sup>1</sup> Tobacco mosaic virus protein contains a very small amount of methionine, 0.16 per cent according to Ross (21), and since our supply of this protein was small, methionine and total sulfur determinations were omitted.

titrating agent, found 0.50 per cent cysteine in edestin, 0.16 per cent in excelsin, less than 0.01 per cent in amandin, 1.19 per cent in rabbit myosin, and 0.34 per cent in serum albumin. These values are almost identical with those reported here. In the globin field considerable variation has been found in the total sulfur, cystine, and methionine content of hemoglobins (30) and of the globins (31) from different species. In the calf globin, we found little cysteine, whereas Greenstein (8) found 0.56 per cent cysteine in denatured horse globin. It is interesting that our value for the total cystine and cysteine content of calf globin is 0.58 per cent, practically identical with Greenstein's total cysteine value in horse globin denatured by guanidine hydrochloride. In the case of myosin, denatured and dried, all of the cysteine complexes were oxidized to cystine complexes and are found as cystine after hydrolysis. As may be seen from Table I, the amount of cysteine found in the acid hydrolysates of these proteins compares closely with the cysteine calculated by titration of the SH groups of the unhydrolyzed native proteins. Accordingly it would appear that the SH groups thus titrated are present as cysteine complexes which react with iodine as cysteine does. This conclusion has some bearing on the mechanism of denaturation, a question that will be discussed in another paper. Anson (25) in fact suggests that the groups which react with iodine are either SH groups or groups which become SH groups when the protein is denatured.

The cysteine, cystine, and methionine content of the proteins accounts for over 90 per cent of the total sulfur in seven out of ten proteins, with an average value of 100.1 per cent. Three of the proteins, globin, edestin, and squash seed globulin, yield 85, 82, and 87 per cent respectively of the total sulfur as the sulfur-containing amino acids. In the case of the globin, owing to the small sulfur content, this disparity may be more apparent than real. It may be noted, however, that Beach, Bernstein, Hummel, Williams, and Macy (31) find, by means of different procedures for analyzing for cystine and methionine, that these amino acids account for about 87 per cent of the total sulfur of beef globin. Since the supply of edestin was limited, this problem was investigated further with the squash seed globulin. Hydrolysis of the squash seed globulin with 20 per cent hydrochloric acid yielded no hydrogen sulfide, nor was there any sulfate in the hydrolysate. Hydrolysis with 57 per cent hydriodic acid in the presence of potassium hypophosphite and also hydrolysis with an equal mixture of concentrated hydrochloric and formic acids yielded cystine and methionine accounting for 88 and 89 per cent of the total sulfur respectively of the intact protein. The sulfur was then determined in the hydrolysates resulting from the hydrolysis with the various acids, except 6 N sulfuric acid. The average value from six determinations was 0.874 per cent S, which is 88.2 per cent of

the total sulfur of the original protein determined by the same method. The total sulfur of the squash seed globulin hydrolysate, however, is accounted for by cysteine, cystine, and methionine. During hydrolysis there is a loss of 11.8 per cent of the sulfur in a form not yet accounted for. Baernstein (32) found that in most proteins cystine and methionine explained the total sulfur. In the case of edestin, however, he was able to account for only 85.6 per cent of the total sulfur as methionine and cystine. During hydrolysis 14.5 per cent of the sulfur was liberated as hydrogen sulfide. This is in contrast with the present observation that no hydrogen sulfide appeared during the hydrochloric acid hydrolysis of the squash seed globulin. In Baernstein's procedure methylmercaptan arising from methionine would be converted to hydrogen sulfide, which Baernstein found. In the hydrochloric acid hydrolysis no hydrogen sulfide would be liberated from methylmercaptan. In view of Baernstein's accounting for 100 per cent of the total sulfur and our accounting for the total sulfur of the hydrolysate of the squash seed globulin, it is not necessary to conclude that another type of sulfur occurs. Rather the volatile sulfur may be supposed to arise from cystine or cysteine, or more probably from methionine by virtue of the type of complex in the protein in question.

The relation of cystine, cysteine, and methionine sulfur content to the total sulfur is given in Table II for the various proteins as determined after hydrolysis with 20 per cent hydrochloric acid.

#### SUMMARY

The cysteine, cystine, and methionine content of the proteins accounts for practically all of the total sulfur of the unhydrolyzed proteins in seven out of ten cases. In three proteins, calf globin, edestin, and squash seed globulin, the cysteine, cystine, and methionine account for respectively 85, 82, and 87 per cent of the total sulfur of the unhydrolyzed proteins. The sulfur-containing amino acids, however, account for all of the sulfur in the hydrolysates of the squash seed globulin. About 13 per cent of the sulfur is lost during the hydrolysis of this globulin in a form not yet explained.

As determined by direct titration of the unhydrolyzed protein with iodine, the total SH found agrees with the cysteine determined in the acid hydrolysate of the same protein. This finding indicates that cysteine complexes are present in the native protein.

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# THE DETERMINATION OF CYSTEINE AND CYSTINE BY VASSEL'S METHOD<sup>1</sup>

By DALE K. MECHAM

*(From the Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)*

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Vassel's method<sup>1</sup> for the determination of cysteine depends upon the development of a blue color by reaction with *p*-aminodimethylaniline, ferric iron, and zinc. A separate preparation of the cysteine reagent is required for each sample. We have found it more convenient to replace these with aliquots of a single large preparation, thereby also eliminating the differences within a series resulting from variations in the color-producing capacity of the separate preparations. Furthermore, variations between the larger lots of reagent were decreased when the ferric iron solution was first reduced with zinc and then combined with the *p*-aminodimethylaniline solution. More precise results have been obtained by the use of these modifications.

For the determination of cysteine plus cystine by the modified procedure, the reduction of cystine requires an additional separate step but results of increased precision are obtained.

Unsatisfactory results were obtained with Vassel's procedure for cysteine plus cystine when detergents (of the alkyl sulfate or alkylaryl sulfonate types) were present in samples of keratin derivatives. The modified procedure gave as consistent results as were obtained in the absence of the detergents (Table I).

## EXPERIMENTAL

The reagents conformed to Vassel's specifications. In addition, granulated zinc (30 mesh, reagent grade) was used.

A photoelectric colorimeter (Wilkens-Anderson KWSZ) fitted with filters giving maximum transmission of light at 5800 to 5900 Å. was used to measure the intensity of the blue color developed. As Vassel reported, the final concentration of zinc affects the intensity of the blue color obtained. With the procedures to be described, 330 mg. of zinc were used in each determination, or twice the quantity used by Vassel. The increased amount gave intensities of color more suitable for measurement in the colorimeter.

*Preparation of Cysteine Reagent*—The following preparation is sufficient for nine separate determinations; larger or smaller amounts can be prepared

<sup>1</sup> Vassel, B., *J. Biol. Chem.*, **140**, 323 (1941).

similarly. To 20 ml. of ferric ammonium sulfate solution in a flask fitted with a condenser, add 1.00 gm. of zinc dust and let it react for 10 minutes with occasional shaking. Add 2.30 gm. of granulated zinc and 30 ml. of

TABLE I

*Determinations of Cysteine Plus Cystine on Hydrolysates of Keratin-Detergent*  
75 mg of keratin plus 10 mg. of Naeconol NRSF.

Keratin	Per cent cysteine plus cystine in keratin	
	Vassel's procedure	Modified procedure
Human hair	14.3, 16.2, 17.0, 15.0	15.7, 16.0, 15.7, 15.3
"    "    treated with NaHSO <sub>3</sub> and urea	15.0, 12.8, 12.3, 12.8	13.5, 13.5, 13.5, 13.5
Chicken feathers	6.7, 5.9, 6.5, 6.7	6.8, 6.9, 6.6, 6.6
"    "    treated with NaHSO <sub>3</sub> and urea	6.3, 5.5, 5.7, 5.9	6.0, 6.0, 6.1, 6.1

TABLE II

*Determinations of Cysteine Plus Cystine on Hydrolysates of Feathers and Hoof*  
Containing Added Cystine

Hydrolysate*		Cysteine plus cystine from hydrolysate	Added cystine	Total cysteine plus cystine	
				Calculated	Found
Feathers	1 ml. diluted to 5 ml.	mg per ml	mg per ml	mg per ml	mg per ml.
	1 " " " 5 "	0.041	0.050	0.091	0.087
	1 " " " 5 "	0.041	0.100	0.141	0.136
	1 " " " 5 "	0.041	0.150	0.191	0.185
	2 " " " 5 "	0.082	0.050	0.132	0.132
Hoof	2 " " " 5 "	0.082	0.100	0.182	0.182
	1 " " " 5 "	0.036	0.050	0.086	0.086
	1 " " " 5 "	0.036	0.100	0.136	0.139
	1 " " " 5 "	0.036	0.150	0.186	0.191
	2 " " " 5 "	0.072	0.050	0.122	0.123
	2 " " " 5 "	0.072	0.100	0.172	0.173

\* 75 mg. of keratin hydrolyzed in 6.25 ml. of HCl-HCOOH reagent; diluted to 25 ml. with 5 N HCl.

*p*-aminodimethylaniline solution and, after an additional 10 minutes, heat the preparation in a boiling water bath for 25 minutes. Cool and filter.

*Preparation of Cysteine Plus Cystine Reagent*—Use only 2.00 gm. of granulated zinc; otherwise follow the procedure for preparation of the cysteine reagent. Less zinc is used here to allow for the zinc dissolved in the samples. The final concentration of zinc in the sample plus reagent

is then the same for cysteine and cysteine plus cystine determinations, permitting the use of the same calibration curve for both.

*Reduction of Cystine for Cysteine Plus Cystine Determinations*—To 5 ml. of hydrolysate (or  $X$  ml. diluted with acid<sup>1</sup> to 5 ml. if necessary to reduce the cysteine plus cystine concentration below 0.2 mg. per ml.) in a test-tube, add 150 mg. of zinc dust. Loosely stopper the tube and allow to stand. After most of the zinc is dissolved, carefully tilt and rotate the tube to dissolve any zinc remaining on the walls of the tube.

*Completion of Determinations*—1 ml. samples of unreduced or reduced hydrolysates are added to 5 ml. portions of cysteine reagent or cysteine plus cystine reagent respectively in 15 × 150 mm. test-tubes, and the reagent and sample are mixed. Determinations are then completed as described by Vassel, beginning with the addition of 3 ml. of ferric ammonium sulfate solution.

A number of separate determinations were run on cystine solutions of known concentration. For twenty determinations (not more than two with a single reagent preparation) the average deviation from expected values was  $\pm 1.4$  per cent for the modified procedure and  $\pm 2.6$  per cent for the Vassel procedure. Vassel reported  $\pm 3$  per cent as the average variation of triplicates from their mean.

Similar results were obtained with cysteine.

Cysteine plus cystine determinations on hydrolysates of feathers and hoof containing added cystine are reported in Table II. Hydrolysates of feathers were found to react more rapidly with zinc dust than hydrolysates from other keratins; the values in Table II were obtained on samples that were allowed to react only 25 minutes. The values on hydrolysates of hoof were obtained on samples that had reacted 60 minutes.

#### SUMMARY

Increased precision in the determinations of cysteine and cysteine plus cystine, particularly in the presence of detergents, has been obtained by modification of some details of Vassel's procedure.



# ASSIMILATION OF CARBON DIOXIDE BY THE ISOLATED MAMMALIAN HEART\*

BY VICTOR LORBER,† ALLAN HEMINGWAY, AND A. O. NIER

(From the Departments of Physiology and Physics, University of Minnesota, Minneapolis)

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The demonstration by Wood and coworkers that  $\text{CO}_2$  can be assimilated by the heterotrophic bacteria (1, 2) was followed by studies from their own and other laboratories showing that the tissues of higher animal forms (including mammals) are capable of fixing  $\text{CO}_2$  (3-6).

When fasted rats are fed glucose or lactate, and injected with isotopic  $\text{CO}_2$  as bicarbonate, the glycogen laid down in the liver contains appreciable quantities of the tagged  $\text{CO}_2$  carbon. Smaller amounts appear in the skeletal muscle (5, 6). That  $\text{CO}_2$  carbon can in some way be incorporated into the glycogen molecule by the liver is evident from these and other studies (3). However, whether muscle tissue can carry out this process independently or must rely on the liver for the initial fixation is a question which cannot be answered by studies on the intact animal. Accordingly, the problem was investigated in the completely isolated cat heart.

## Methods

Experiments were carried out with the completely isolated cat heart, by means of a modification of a technique previously described (7). The open oxygenator was ventilated for a period of about 15 minutes with a brisk stream of pure oxygen to reduce the blood  $\text{CO}_2$  content. The respiratory circuit was then closed for the rest of the experiment, usually  $1\frac{1}{2}$  hours. To the respiratory gas was added enough  $\text{CO}_2$  containing approximately 9 per cent  $\text{C}^{13}$  to bring the initial level of  $\text{CO}_2$  to 4 to 5 volumes per cent. The soda lime chamber was omitted, and a large spirometer substituted for the small one ordinarily used. The total gas volume of the system remained fairly constant throughout, and was fixed at such a magnitude that as  $\text{CO}_2$  accumulated its concentration rose by no more than 1 to 2 volumes per cent. This was checked by analysis.

During the course of an experiment,  $\text{C}^{13}$  was also added to the blood in the

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† This work is part of a thesis submitted to the Faculty of the Graduate School of the University of Minnesota by Victor Lorber in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

form of a carbonate-bicarbonate solution containing approximately 1 mm of  $\text{CO}_2$  (9 per cent  $\text{C}^{13}$ ) per cc. About 4 to 5 cc. of solution were injected, being first diluted 1:1 with Ringer's fluid and then administered slowly in four or five equally spaced doses. In one experiment in which measurements were made, the blood pH rose from 7.2 to 7.5 as a result of the added alkali.

Five experiments were carried out. In two, glucose and sodium lactate were added to the blood, in one, glucose, and in two, glucose and insulin. These measures were taken in an effort to favor glycogen deposition (8-10).

At the close of an experiment the heart was rapidly removed from the perfusion system and the cardiac glycogen isolated according to the method of Good, Kramer, and Somogyi (11). Hydrolysis was carried out with  $\text{N H}_2\text{SO}_4$  and glucose determined on an aliquot with the Shaffer-Hartmann reagent. The remainder of the glucose was oxidized to  $\text{CO}_2$  by the method

TABLE I  
*CO<sub>2</sub> Fixation in Tissue Glycogen in Cardiac Muscle*

$\text{C}^{13}/\text{C}^{12} \times 100 = 1.100$  was used as a standard.

Experiment No.	Procedure	Cardiac glycogen*	$\frac{\text{C}^{13}}{\text{C}^{12}} \times 100$ in cardiac glycogen	Per cent cardiac glycogen* containing $\text{CO}_2$ carbon
1	Control glycogen analysis	109	1.055	
2	" " " following <i>in vitro</i> contamination with $\text{C}^{13}$	109	1.084	
3	$\text{C}^{13}$ + glucose and lactate	140	1.260	9
4	" + " " "	147	1.260	14
5	" + " lactate, and insulin	149	1.370	24
6	" + " and insulin		1.400	27
7	" + " " "	151	1.260	14

\* Expressed as mg. of glucose per 100 gm. of heart muscle.

of Van Slyke and Folch (12) and analyzed for its  $\text{C}^{13}$  content with the mass spectrometer (13). The isotopic carbon was prepared according to the method described by Nier (14). The  $\text{CO}_2$  content of the NaOH used for collecting the gas evolved from the combustion of glucose was determined in the manometric Van Slyke apparatus, in order to correct for dilution of the sample by  $\text{CO}_2$  of normal isotopic content.

A control sample of heart muscle was heavily contaminated *in vitro* with  $\text{C}^{13}$  (carbonate-bicarbonate solution) to test the efficacy of the glycogen isolation in eliminating  $\text{CO}_2$  present in inorganic form.

### Results

The results of all experiments are presented in Table I. It will be noted that in the glycogen from all of the experimental hearts the abundance of

$C^{13}$  is significantly higher than the value for naturally occurring carbon (around 1.1 per cent for inorganic carbon, and 1.06 per cent for carbon from animal sources). The small difference noted between the uncontaminated and contaminated controls (Experiments 1 and 2) may indicate failure to remove the added  $C^{13}$  completely, but certainly shows that the method was adequate, particularly since combustion was carried out directly on the glycogen in the control experiments, and on the acid hydrolysate in the others.

The fraction of the total cardiac glycogen containing  $CO_2$  carbon may be roughly estimated from the above results. Assuming that 1 molecule of  $CO_2$  is utilized for each molecule of glucose deposited as glycogen, the percentage of the cardiac glycogen containing  $C^{13}$  can be readily calculated. If it is further assumed that the normal  $CO_2$  and isotopic  $CO_2$  react in proportion to their respective concentrations, approximate values for the fraction of the total cardiac glycogen into which  $CO_2$  carbon has been incorporated may be arrived at. In making the calculations, the value for the ratio of the concentration of  $C^{13}$  to  $C^{12}$  has been set at 1:13. This is based on average figures for perfusion blood volume, blood bicarbonate, and added  $C^{13}$ . In this way, the fraction of cardiac glycogen containing  $CO_2$  carbon has been found to range from 9 to 27 per cent. These figures are particularly striking when one considers the length of the experiments,  $1\frac{1}{2}$  hours. (In the calculations, the abundance of  $C^{13}$  from inorganic sources has been used as the normal, thus adding to the significance of the results.)

#### DISCUSSION

These results are of interest in a number of connections. They show that  $CO_2$  fixation in tissue glycogen occurs in mammalian cardiac muscle as well as in liver, and suggest that it is a fundamental reaction in carbohydrate metabolism.

Considering the similarity of the results obtained with and without added insulin, and the relatively low total glycogen values observed, it seems fair to conclude that the glycogen of working cardiac muscle is constantly being broken down and reconstituted, a view previously expressed by Cruickshank on the basis of other evidence (15).

#### SUMMARY

Completely isolated, working, mammalian cardiac muscle fixes  $CO_2$  carbon in the tissue glycogen.

The possible significance of this observation is indicated.

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# INHIBITION OF BRAIN RESPIRATION BY PICROTOXIN

BY J. RAYMOND KLEIN

WITH THE TECHNICAL ASSISTANCE OF MARVIN HACK

(From the Departments of Psychiatry and Physiological Chemistry, University of Illinois College of Medicine, Illinois Neuropsychiatric Institute, Chicago)

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Although the stimulatory effect of picrotoxin on the central nervous system is well known, there is little information concerning the effect of the drug on the metabolism of brain. It has been stated (1, 2) and denied (3) that the convulsions induced by picrotoxin result in a decrease in the concentration of cerebral glycogen. The concentration of lactic acid in brain was found to increase during convulsions to an extent presumably not attributable to the increased muscular activity (4). The oxygen tension of the cerebral cortex decreases just prior to the onset of the increased electrical activity induced by picrotoxin and other convulsant drugs (5). This decrease in oxygen tension suggests that a change in cerebral metabolism occurs just prior to the onset of the convulsive state.

In the present work it was found that the respiration of a cat brain preparation and the oxidation of glutamate, succinate, fumarate, and pyruvate by the preparation *in vitro* were inhibited by picrotoxin.

## Methods and Materials

The brain was prepared as follows: A cat was killed by a blow on the head and decapitation. The brain was removed and homogenized with 30 ml. of 0.04 M potassium phosphate, pH 8.0, containing 0.02 M magnesium chloride, and then squeezed through muslin. The pH of the mixture was about 7.0. In all experiments 1 ml. of this preparation and 0.2 ml. of substrate solution or water were diluted to a final volume of 2 ml. with a solution that contained enough of the following substances to give the final concentrations indicated: 0.08 M nicotinamide, 0.0012 M diphosphopyridine nucleotide, 0.0008 M thiamine pyrophosphate, 0.0016 M sodium fumarate, 0.0012 M adenosine triphosphate, 0.036 M sodium fluoride, and 0.024 M potassium phosphate, pH 8.0. The pH of the final preparation was about 7.2.

The purity of the diphosphopyridine nucleotide was 0.5, as estimated from nicotinic acid and phosphate analyses; of the adenosine triphosphate 0.95, as indicated by total phosphate and phosphate hydrolyzable in 7 minutes by 1 M hydrochloride at 100°. The thiamine pyrophosphate was

supplied by Merck and Company, Inc. The picrotoxin, which was usually added to the experimental mixtures as the solid, was obtained from the Inland Alkaloid Company.

The oxygen uptake of the brain preparations and the oxidation of the substrates employed were followed manometrically at 37° in air with the usual Warburg apparatus. Inorganic phosphate (6), lactate (7), and fructose (8) were estimated colorimetrically. Phosphoglycerate was assayed as acid-soluble phosphorus not hydrolyzed in 180 minutes by 1 N sulfuric acid at 100°. Bisulfite binding was used as a measure of pyruvate (9).

*Effect of Picrotoxin on Respiration of Brain*—The data in Fig. 1 illustrate the inhibitory effect of picrotoxin on the oxygen uptake of the brain prep-

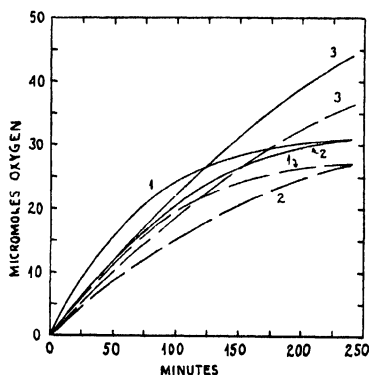


FIG. 1. Inhibition of oxygen uptake of brain by  $4 \times 10^{-3}$  M picrotoxin. The continuous and broken lines represent the uptake without and with picrotoxin respectively. Curves 1, uptakes of the brain preparation described in the text; Curves 2, uptakes of the same preparation without adenosine triphosphate; Curves 3, uptakes without adenosine triphosphate and fluoride.

aration under various conditions. In the absence of fluoride the extent of inhibition remained relatively constant at about 15 per cent. In the presence of fluoride alone and with fluoride plus adenosine triphosphate the extent of inhibition reached a maximum of about 35 per cent, but changed with time. With adenosine triphosphate and fluoride the maximum inhibition developed earlier than with fluoride alone. The extent of inhibition in other experiments was of the same order, and the times at which maximum inhibition developed were relatively the same.

The following considerations give some indications concerning the substances being oxidized in the preparations and permit a limited localization of the inhibitory effect of picrotoxin. The respiratory quotient obtained without fluoride was 0.90, indicating that the oxygen uptake was due, at least in part, to oxidation of carbohydrate. In such preparations, however,

a considerable fraction of the oxygen uptake may be due to oxidation of non-carbohydrate substances (10). Fluoride affects the oxidation of carbohydrate in part by preventing the further conversion of phosphoglycerate, and might be expected to limit the oxygen uptake to that required for the oxidation of triose phosphate, preformed lactate and pyruvate, and possibly hexose monophosphate. Oxygen uptake due to oxidation of lactate and pyruvate can be disregarded, since the complete oxidation of the initial amount of these substances in the preparations containing fluoride accounts for less than 5 per cent of the oxygen uptake. Adenosine triphosphate is required for the phosphorylation of carbohydrate that precedes oxidation and might be expected to increase the rate of formation of hexose and triose phosphate, with a consequent increase in rate of oxygen uptake. In agreement with these possibilities, the data in Fig. 1 indicate that fluoride limited the oxygen uptake and that adenosine triphosphate increased the initial rate of uptake. It seems reasonable to assume, therefore, that the oxygen uptake of the preparations containing fluoride was due in part to an oxidation of triose phosphate. Compatible with this assumption is the fact that phosphoglycerate accumulated in the preparations containing fluoride. Therefore, the inhibitory effect of picrotoxin on the oxygen uptake of the brain preparation may be due in part to inhibition of the oxidation of triose phosphate.

Oxidation of triose phosphate in tissue preparations depends upon phosphorylation for the formation of hexose diphosphate, the immediate precursor of the triose. Phosphorylation is coupled with the conversion of the initial product of oxidation, diphosphoglycerate, to phosphoglycerate (11). Inhibition of triose phosphate might be effected, therefore, through interference with phosphorylation. The data in Table I show that picrotoxin inhibited the phosphorylation of glucose, as indicated by the decreased removal of inorganic phosphate and decreased formation of fructose. The data also show that the phosphorylation was quantitatively more sensitive to the presence of picrotoxin than the oxygen uptake.

*Oxidation of Pyruvate*—Fig. 2 illustrates the effect of picrotoxin on the oxidation of pyruvate by the brain preparation. The extent of inhibition was apparently greater with the preparation without fluoride and adenosine triphosphate. Comparison of the data in Fig. 2 with the oxygen uptakes of the controls which are given in Fig. 1 shows that an appreciable increment in oxygen uptake in the presence of pyruvate was not obtained until the rate of uptake in the control had decreased considerably. Therefore, it appears that the inhibition obtained may have been due only to a delay in the oxidation of the substrates in the control. In order to test this possibility, an experiment was carried out in which pyruvate and picrotoxin were added after the control uptake had decreased to a comparatively slow rate. The data obtained are given in Fig. 3.

The data show that picrotoxin inhibited the oxidation of pyruvate when the rate of the control uptake was relatively small, and the effect of picrotoxin on the control rate was negligible. Therefore, the effect of picrotoxin on the rate of oxidation was primary; *i e.*, the effect was not due to a competition between pyruvate and the preformed substrates in the control for the necessary enzyme systems.

The pattern of pyruvate oxidation did not seem to be affected by picrotoxin. This was indicated by the same R.Q. values in the presence and absence of picrotoxin, the values obtained, 1.3 to 1.4, being somewhat higher than the theoretical 1.25 for complete oxidation of pyruvate. The inhibition of oxidation was probably not due to diversion of the pyruvate

TABLE I

*Effect of  $5 \times 10^{-3}$  M Picrotoxin on Phosphorylation of Glucose by Brain*

The preparation of brain used is described in the text. The concentration of glucose was 0.02 M. The mixtures, after incubation in air for 65 minutes, were treated with 1 ml. of 2 M trichloroacetic acid, diluted to 10 ml., and then filtered. The filtrates were analyzed colorimetrically for inorganic phosphate and fructose. The fructose found is presented in terms of fructose-1,6-diphosphate (12).

Experiment No.	Conditions	Oxygen uptake	Decrease in inorganic phosphate	Formation of fructose diphosphate
		<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
1	Control	19	11	0
	" + picrotoxin	11	7	0
	Glucose	18	72	23
	" + picrotoxin	13	27	3
2	Control	13	10	0
	" + picrotoxin	8	1	0
	Glucose	12	59	17
	" + picrotoxin	9	14	0

into non-oxidative channels, since the amount of pyruvate remaining after the experiments was inversely proportional to the amount disappearing due to oxidation.

Among other substances, oxidation of pyruvate by pigeon brain is effected by adenosine-5-phosphates, fumarate, and diphosphopyridine nucleotide (13). Data showing the effect of adenosine triphosphate, diphosphopyridine nucleotide, and fumarate on the inhibition of pyruvate oxidation by picrotoxin are given in Fig. 4. Neither nucleotide changed the extent of inhibition. Fumarate decreased the extent of inhibition from 45 to 30 per cent. Although fumarate did not increase the rate of oxidation in the absence of picrotoxin in all experiments, it did increase the rate of oxidation in the presence of picrotoxin.

It may be pointed out that the evidence for participation of the pyridine nucleotide in pyruvate oxidation as an oxidant is equivocal, since it was found that the nucleotide could serve as a substitute, but not as a supplement, for the adenosine phosphate (13). Comparison of the data in Figs. 3 and 4 and the results of other experiments indicates that the rate of oxidation of pyruvate by the brain preparation was not limited by the concen-

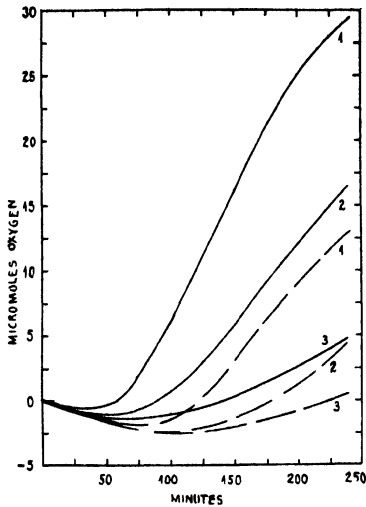


FIG. 2

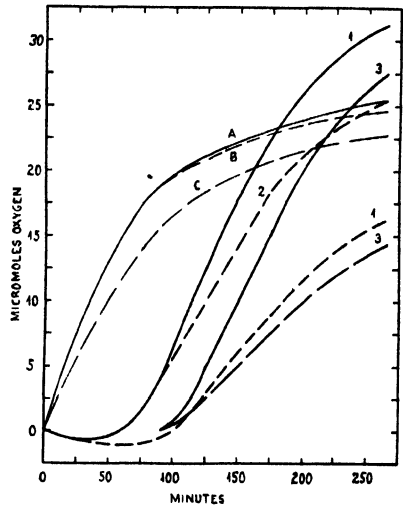


FIG. 3

FIG. 2. Effect of  $4 \times 10^{-3}$  M picrotoxin on the oxidation of pyruvate by brain. The data given represent the differences between the oxygen uptakes with and without 0.01 M pyruvate. The continuous and broken lines represent uptakes without and with picrotoxin respectively. Curves 1, the brain preparation described in text; Curves 2, the same preparation without adenosine triphosphate; Curves 3, the preparation without fluoride and adenosine triphosphate.

FIG. 3. Inhibition of pyruvate oxidation by  $4.1 \times 10^{-3}$  M picrotoxin. The oxidation of 0.01 M pyruvate is represented by the differences between the oxygen uptakes with and without pyruvate. The continuous and broken lines represent uptakes without and with picrotoxin respectively. Curves A and C, the preparation described in text; Curve B, with picrotoxin added at 90 minutes; Curves 1, with pyruvate and picrotoxin added at 0 minutes; Curve 2, with pyruvate added at 0 minutes, picrotoxin at 90 minutes; Curves 3, with pyruvate and picrotoxin added at 90 minutes.

tration of adenosine triphosphate. The data in Fig. 4 show that diphosphopyridine nucleotide in the presence of excess adenosine phosphate increased the rate of oxidation of pyruvate. This signifies that diphosphopyridine nucleotide serves as an oxidant in the oxidation of pyruvate.

It appears then that picrotoxin inhibits the oxidation of pyruvate by brain, and that the inhibitory effect may be upon fumarate catalysis. The

effect of picrotoxin on pyruvate oxidation may account in part for the inhibition of the respiration of the brain preparations alone.

*Oxidation of Glutamate, Succinate, and Fumarate*—Data illustrating the effect of picrotoxin on the oxidation of glutamate, succinate, and fumarate by the brain preparation are given in Fig. 5. In this experiment the extent of inhibition at 50 and 100 minutes was practically the same for all

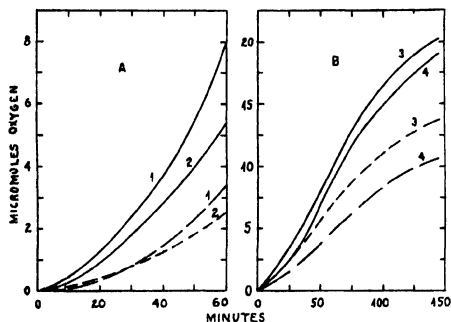


FIG. 4

FIG. 4. Effect of diphosphopyridine nucleotide and fumarate on the oxidation of pyruvate. The brain preparations were made as described in the text. One (A) was incubated aerobically 215 minutes and the other (B) was incubated 120 minutes, at which times the substances indicated were added in sufficient amount to increase the initial concentrations to the concentrations given. The concentration of pyruvate was  $0.01\text{ M}$ . The oxidation is represented by the differences between the oxygen uptakes with and without pyruvate. The continuous and broken lines represent the uptakes without and with  $4.1 \times 10^{-3}\text{ M}$  picrotoxin. Curves 1,  $5 \times 10^{-3}\text{ M}$  diphosphopyridine nucleotide; Curves 2, with and without  $3 \times 10^{-3}\text{ M}$  adenosine triphosphate; Curves 3 and 4, with and without  $10^{-3}\text{ M}$  fumarate respectively.

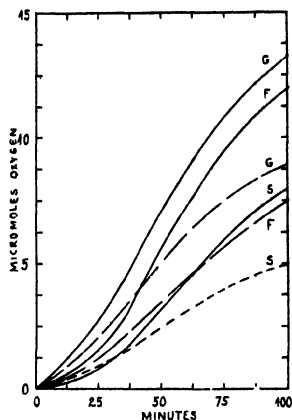


FIG. 5

FIG. 5. Effect of  $4.1 \times 10^{-3}\text{ M}$  picrotoxin on the oxidation of glutamate, succinate, and fumarate, by brain. The concentration of substrates was  $0.01\text{ M}$ . The preparation described in the text was incubated 125 minutes before addition of the substrates. The continuous and the broken lines represent the uptakes without and with picrotoxin respectively. Curves G, glutamate; Curves S, succinate; Curves F, fumarate.

of the substrates. The effect of picrotoxin depends upon the preparation of brain used. As indicated previously for pyruvate (Fig. 2) the rate and extent of oxidation of these substrates are increased markedly in the presence of fluoride and adenosine triphosphate. In the absence of the two latter substances the oxidation of glutamate, as indicated by the oxygen uptake, carbon dioxide, and ammonia production, appears to stop with the formation of fumarate. Likewise the oxidation of succinate, as indicated by the

oxygen uptake and estimation of residual succinate by oxidation with washed muscle (14), stops with the formation of fumarate. The oxidation of glutamate and succinate under these conditions was not appreciably affected by picrotoxin. The R.Q. for the oxidation of these substances by brain preparations containing fluoride and the adenosine phosphate indicates complete oxidation. Therefore, it appears that the effect of picrotoxin on the oxidation of glutamate and succinate was probably due to an inhibition of the oxidation of fumarate.

#### DISCUSSION

It is not possible to state whether the inhibition of brain respiration *in vitro* is concerned in the stimulatory effect of picrotoxin *in vivo*. The effective convulsive dose of picrotoxin for cats is of the order of  $5 \times 10^{-3}$  mm. The concentration of the drug at the site where it produces its effect is not known. Under the conditions of the experiments described the dose indicated will inhibit the oxidation of pyruvate and fumarate by 0.5 gm. of brain to the extent of 50 per cent.

#### SUMMARY

The respiration of a cat brain preparation and the oxidation of glutamate, succinate, fumarate, and pyruvate by the preparation *in vitro* was inhibited by picrotoxin. The effect of the drug on glutamate and succinate was probably due to an inhibition of the oxidation of fumarate or pyruvate, rather than upon the first stages in the oxidation of the former substances. The effect of picrotoxin upon the oxidation of pyruvate may have been due to inhibition of fumarate catalysis, since fumarate decreased the extent of inhibition.

The laboratory is indebted to Merck and Company, Inc., for the thiamine pyrophosphate used in these experiments.

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# PREPARATION AND ANTIGENIC PROPERTIES OF A CRYSTALLINE LABELED ANTIGEN

By BAHMAN K. SHAHROKH

(From the Department of Bacteriology, University of California, Berkeley)

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In certain types of studies in the field of immunology there is a need for a labeled antigen which can be easily prepared and purified. Artificially conjugated proteins have been used as labeled antigens, but the methods used for their purification have not been very satisfactory. Therefore, it was thought advisable to investigate the possibilities of preparing a traceable protein which could be purified by crystallization. A number of experiments were carried out with iodoalbumins of low iodine content and it was found that iodinated horse serum albumin containing less than 4.4 per cent iodine could be crystallized. In this paper the methods of preparation and crystallization, as well as the antigenic properties of this artificially conjugated protein, are discussed.

## *Preparation of Crystallizable Iodoalbumin*

Preliminary experiments indicated that the rate of oxidation and iodination of albumin by iodine is dependent on four factors: (1) the ratio of added iodine to nitrogen, (2) the concentration of protein (for the same ratio of added iodine to nitrogen, lower iodination and oxidation values are obtained with protein solutions of higher concentrations), (3) the temperature of incubation (greater oxidation values are obtained when higher temperatures are used for incubation), and (4) the period of incubation.

It was found that albumin crystallized three times would give satisfactory and constant results. The albumin was crystallized according to the method of Hopkins as modified by Young (1). Recrystallization was carried out according to the method of Adair and Robinson (2). The crystals were then centrifuged and dissolved in phosphate buffer at pH 6.5, and dialyzed against distilled water in a rocking dialyzer for 24 hours. Nitrogen determinations were made on the protein, and the nitrogen content was brought to 1.5 mg. of N per ml. by the addition of phosphate buffer<sup>1</sup> and water.

<sup>1</sup>50 gm. each of anhydrous disodium hydrogen phosphate and potassium dihydrogen phosphate were dissolved in 500.0 ml. of water and filtered. The pH of this solution as determined with the aid of the glass electrode was found to be 6.54. For experimental purposes, 20 ml. of this buffer were used per 100 ml. (final volume) of the albumin and control solutions.

10 ml. of iodine in potassium iodide solution were added to 100 ml. of buffered albumin solution which had been cooled to 5°. Fig. 1 shows the concentrations of iodine which should be used for obtaining iodoalbumins of desired iodine contents. Phosphate controls were run at the same time. Glass-stoppered bottles were used in the preparation and the mixtures were left at 5° for 15 hours. Iodometric titrations were made on 5 ml. samples of both control and albumin solutions and the free iodine in the main reaction mixture was reduced by the addition of sufficient saturated sodium thiosulfate solution. The iodoalbumin was then precipitated by the addition of an equal volume of saturated ammonium sulfate solution and enough 10 per cent acetic acid to make it distinctly turbid. The mixture was left in the ice box for 2 to 3 hours, after which the precipitate was centrifuged and dissolved in 100 ml. of 0.3 M sodium acetate. To this was

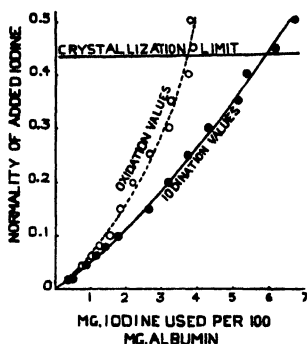


FIG. 1. Oxidation and iodination of horse serum albumin by iodine solutions of different concentrations for preparation of crystalline iodoalbumin

added an equal volume of saturated ammonium sulfate solution followed by 3 ml. of 1 N acetic acid. The mixture was then allowed to stand for 15 minutes, and 2 ml. portions of 1 N acetic acid were added at 15 minute intervals until a very faint turbidity appeared within 15 minutes after the addition. The solution was then left overnight at room temperature. A better yield of crystals could be obtained if 10 ml. of ammonium sulfate solution were slowly added to the mixture 2 hours after the crystallization had started. The flasks had to be shaken frequently, especially after the addition of the reagents.

Varying quantities of acetic acid were needed for iodoproteins with different iodine contents. With proteins of higher iodine content, less acetic acid was needed, and crystallization had to be carried on with greater caution, the additions of acetic acid being made more slowly and at intervals longer than 15 minutes. In the majority of cases a considerable amount of amorphous protein settled with the crystals when the iodine

content was about 3.35 per cent or greater. In order to convert this amorphous substance into crystals, the solution was kept at room temperature for several days.

In order to recrystallize, the crystals were centrifuged and dissolved in 75 ml. of a 0.3 M solution of sodium acetate and the crystallization procedure was repeated, the quantities of the reagents being proportionately reduced. The iodine content of iodoalbumin did not change upon recrystallization.

### *Methods*

The iodine content of the iodoproteins was determined as follows: After the iodometric titration, the iodoalbumins were placed in cellophane bags and dialyzed for 24 hours against running distilled water. The next day the bags were punctured and the contents emptied into 50 ml. centrifuge tubes. The solutions were heated until the protein was coagulated. During this process the iodine content of the protein does not alter. The precipitate was centrifuged and washed with 20 ml. of water. It was then dissolved in 5.0 ml. of sulfuric acid (sp. gr. 1.84, diluted with an equal volume of distilled water). Nitrogen and iodine determinations were carried out on 2 ml. aliquots of these solutions. The chlorate digestion method (3) which was especially developed for this type of work was used for the determination of iodine.

The amount of iodine bound to 100 mg. of albumin was calculated with the use of the factor 6.25 for the protein-nitrogen ratio. The total amount of iodine reduced by 100 mg. of the protein was similarly computed from the iodometric titration. From this pair of values the number of mg. of iodine consumed in oxidation reactions by 100 mg. of albumin was calculated, as described by Herriott (4).

### *Crystals*

The size and shape of the crystals were found to vary to some extent, depending upon the speed of crystallization. The three general forms of iodoproteins which can be recognized are shown in Figs. 3 to 6. Fig. 2 is a photomicrograph of horse serum albumin given for comparison. These crystal forms overlap, and the gradual changes can be traced from one form to another. The crystals of horse serum albumin are long but with the introduction of iodine in the protein they become shorter (Fig. 3). When the iodine content reaches 1.4 per cent, there is a tendency for two crystals to become attached at their bases. This tendency increases until the iodine content reaches 1.9 per cent, at which point single crystals can be found only occasionally (Fig. 4). With further increase in the iodine content some of the crystals reassume their single form and the blunt ends become more pointed (Fig. 5). Among these crystals one finds larger crystals



FIG. 2

FIG. 2 Crystals of horse serum albumin  $\times 380$ .

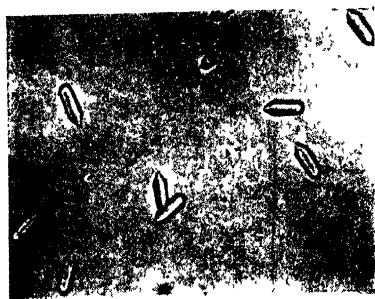


FIG. 3

FIG. 3 Crystals of horse serum iodoalbumin.  $\times 380$ . Iodination value, 0.8 per cent; oxidation value, 0.64 mg of I per 100 mg of albumin



FIG. 4

FIG. 4. Crystals of horse serum iodoalbumin  $\times 380$ . Iodination value, 1.94 per cent; oxidation value, 1.28 mg. of I per 100 mg of albumin.



FIG. 5

FIG. 5. Crystals of horse serum iodoalbumin.  $\times 380$ . Iodination value, 4.41 per cent; oxidation value, 3.53 mg of I per 100 mg of albumin



FIG. 6 Aggregate of crystals of horse serum iodoalbumin.  $\times 380$ . Same composition as in Fig. 5.

which under high magnification appear to be composed of the smaller crystals arranged side by side.

The photomicrography of such large crystals, especially under the 4 mm.

objective, is difficult and certain portions of the crystal are apt to be out of focus. Fig. 6 does, however, show the general arrangement of the aggregation of small crystals.

### *Antigenic Properties of Crystalline Horse Serum Iodoalbumin*

Since the work of Obermeyer and Pick (5) the immunological properties of iodoproteins have received considerable attention. Most of the studies, however, have been made with either whole serum or serum globulin. Therefore it was thought advisable to study the antigenic relationships of

TABLE I

#### *Antigenic Relationship of Horse, Egg, and Bovine Albumins and Iodoalbumins*

— indicates no precipitation, + indicates titers 1:2 to 1:16, ++ titers 1:32 to 1:256, +++ titers 1:512 to 1:1024.

Antigen No.	Source	Iodination value	Oxidation value	Antisera prepared against antigens				
				1	2	3	4	5
1	Horse	0	0	+++	+++	++	+	±
2		0.80	0.65	++	+++	++	++	++
3		1.92	1.30	++	++	++	++	++
4		2.80	1.90	++	++	++	++	++
5		4.38	3.39	++	++	++	++	++
6		7.84	3.84	+	++	++	++	++
7		10.80	7.28	+	++	++	++	++
8		13.05	8.48	+	++	++	++	++
9	Egg	0	0	—	—	—	—	—
10		5.76	8.32	—	+	+	+	+
11		9.28	33.28	—	+	+	+	+
12	Bovine	0	0	—	—	—	—	—
13		7.20	4.48	—	+	+	+	+
14		11.70	8.96	—	+	+	+	+

horse serum iodoalbumins and to determine the specificity changes which would result from various degrees of iodination.

### EXPERIMENTAL

Crystalline iodoalbumins containing different percentages of iodine were prepared. The dialyzed samples were sterilized by filtration and the nitrogen content was adjusted to 2 mg. of N per ml. by the addition of sterile distilled water and sufficient sterile 10 per cent sodium chloride solution to bring the concentration of the salt to that of physiological saline.

Rabbits weighing between 3 and 4 pounds were used for the production of antibodies. Three rabbits were used for each antigen.

The first injection was given intraperitoneally, followed by five intravenous injections. The last injection was administered intradermally. A total of 19 ml. of antigen was used in the course of immunization.

The antigens for precipitation tests were diluted to contain 0.1 mg. of N per ml., and Hanks' method (6) was used for the tests.

### Results

The results obtained are shown in Table I, from which the following conclusions may be drawn. (1) Antisera against horse serum albumin react with all crystalline horse serum iodoalbumins. (2) Antibodies against crystalline iodoalbumins, with the exception of iodoalbumin containing 4.38 per cent of iodine, react with albumin. (3) Crystalline iodoalbumins induce the production of antibodies which react with horse, egg, and bovine iodoalbumins.

### DISCUSSION

Iodoalbumins containing a low percentage of iodine can be used as labeled antigens as long as the species specificity is not of importance in the problem under consideration. The fact that these iodoproteins can be crystallized is a good indication that structurally the protein molecule is not greatly altered, and that the changes which have taken place are more or less uniform. The author believes that an iodoalbumin containing about 2 per cent of iodine would be most suitable for use as a labeled antigen.

### SUMMARY

1. A method for the preparation and crystallization of horse serum iodoalbumins is described. Iodoalbumins containing 4.4 per cent of iodine, or less, can be crystallized.

2. The antigenic properties of crystalline iodoproteins were studied. It was found that these iodoproteins would be of value as labeled antigens if the species specificity is not of importance in the problem under consideration.

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# THE REACTION OF HEMOGLOBIN WITH NITRITE

By LEON A. GREENBERG, DAVID LESTER, AND HOWARD W. HAGGARD

(From the Laboratory of Applied Physiology, Yale University, New Haven)

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Although nitrite has been extensively used as an agent for the formation of methemoglobin, the quantitative relations of the reaction appear to be uncertain; three widely different values have been reported for the amount of methemoglobin formed to nitrite utilized. The first statement of a quantitative relationship was made by Barcroft and Müller (1) in 1911. In a preliminary report containing no experimental data or equations, and for which none was supplied in subsequent publications, they indicated the molar ratio of nitrite utilized to methemoglobin formed as 2; that is, 2 molecules of nitrite react with 1 molecule of hemoglobin to produce 1 molecule of methemoglobin. This relation was accepted by Stadie (2) in 1921 in his investigations on methemoglobin formation. From experimental determinations, Van Slyke and Vollmund (3) in 1925, Meier (4) in 1925, and von Issekutz (5) in 1939 reported that 1 molecule of nitrite was used in forming 1 molecule of methemoglobin; *i.e.*, a molar ratio of 1. Austin and Drabkin (6) in 1935 reported a ratio of 0.5 to 0.7 and one of approximately 0.5 can be calculated from data published in 1942 by Darling and Roughton (7) dealing with the effect of methemoglobin on the oxygen dissociation curve.

The reasons for some of these discrepancies will be dealt with in detail in subsequent discussion. An obvious source of error in several of the investigations was in the assumption that all nitrite added to blood or solutions of hemoglobin was quickly and completely utilized in methemoglobin formation. Analyses were not made for residual nitrite. In the investigation reported here, this source of error was avoided. The amount of nitrite reacting with hemoglobin was determined and the influence of temperature, concentration of nitrite, and of hydrogen ion concentration on the reaction was studied.

## EXPERIMENTAL

Adult white rats were used for experiments *in vivo* and freshly shed heparinized<sup>1</sup> rat blood for experiments *in vitro*. Hemoglobin and methemoglobin were determined by the method of Evelyn and Malloy (8). The concentration of nitrite in the blood was determined by a modification of

<sup>1</sup> Crystalline liquaemin was kindly supplied by the Roche-Organon Corporation.



the method of Stieglitz and Palmer (9) with sulfanilic acid instead of 2-naphthylamine-6,8-disulfonic acid.

To three 2 cc. portions of whole blood containing 8.20 mm of hemoglobin per liter there were added 0.02, 0.04, and 0.06 cc. of a 1.0 per cent solution of sodium nitrite yielding 1.45, 2.90, and 4.35 mm of nitrite per liter in the respective portions. The blood was maintained at 20°. At the end of 30 minutes, 1 hour, and each hour thereafter, determinations were made for

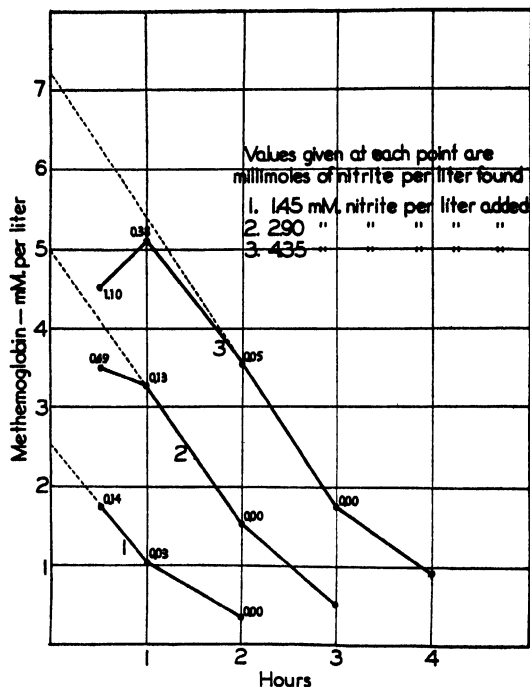


FIG. 1. Methemoglobin and residual nitrite in whole blood after addition of sodium nitrite.

methemoglobin and nitrite. The findings are recorded in Fig. 1. The residual nitrite, as mm per liter, is shown by the small figure at each point plotted.

The curves express the relation between the rate of formation of methemoglobin from the gradually disappearing nitrite and the rate of the continuous reduction of methemoglobin. At the time intervals used, the maximum formation of methemoglobin was found for the two smaller amounts of nitrite at 30 minutes and for the largest at 1 hour. At these times, 9 to 17 per cent of the nitrite was still present; it did not disappear

until the 2nd or 3rd hour. The rate of disappearance of methemoglobin increased as the nitrite concentration approached zero. At the low concentrations of methemoglobin reached by the time all, or nearly all, of the nitrite had disappeared, the rate of reduction slowed, as has been indicated by Gelinsky (10) and Cox and Wendel (11). On the assumption that, except at these low levels, the rate of reduction is at a uniform rate (11), an approximation can be made of the total methemoglobin formation from the nitrite by extending the curve for the maximum rate of reduction back to zero time. These extensions are shown by the dotted lines in Fig. 1; the amounts of methemoglobin indicated by these extrapolations are 2.5, 5.0, and 7.2 mm per liter for 1.45, 2.9, and 4.35 mm of nitrite respectively. The corresponding ratios of nitrite utilized to methemoglobin formed are 0.58, 0.55, and 0.59. In another series of experiments, ratios of 0.55, 0.52, and 0.53 were obtained. The possible sources of error in this extrapolation would tend toward a high rather than a low ratio: (a) during the period of maximum rate of reduction of methemoglobin a small amount of nitrite was still present; (b) the rate of reduction from which the extrapolation was made occurred when the reduction was more than half complete and if, as reported by Gelinsky (10), but contrary to Cox and Wendel (11), the rate of reduction varies with the concentration, the slope of the curve expresses a rate less than the average.

In the second series of experiments, laked blood was employed in order to eliminate the reduction of methemoglobin. To each of two portions of red cells laked with distilled water and containing 6.65 mm of hemoglobin per liter, 2.84 mm per liter of sodium nitrite were added. One portion was kept at 20° and the other at 37°. At 30 minutes, 1 hour, and each hour thereafter, methemoglobin was determined, and at 5 hours, the residual nitrite. The findings are given in Fig. 2. The rate of methemoglobin formation was more rapid at 37° than at 20°; at 5 hours the respective concentrations were 4.5 and 2.75 mm. The residual nitrites were 0.57 and 1.50 mm per liter. The molar ratios of methemoglobin formed to nitrite utilized were 0.49 and 0.51. Thus, although the rate of methemoglobin formation in laked blood is markedly influenced by temperature, the molar ratio of the reaction of hemoglobin and nitrite is not affected.

In a third series of experiments, sodium nitrite was added to each of six portions of red cells laked by water or blood laked by saponin in amounts sufficient to give molar ratios of 0.5 to 1.05 with the hemoglobin present. The temperature was 20°. At 1 and 3 hours, determinations were made of methemoglobin and residual nitrite. The molar ratios of methemoglobin formed to nitrite used, Table I, at both times were between 0.48 and 0.57; they were not influenced by the concentration of nitrite present.

In a fourth series of experiments, nitrite was added to blood laked by

saponin in quantities to give molar ratios of 0.15 to 1.16 with the hemoglobin present; potassium dihydrogen phosphate was then added to make the blood acid and the concentrations of methemoglobin and nitrite were determined 3 and 5 minutes later. These short intervals were employed to avoid appreciable autoxidation of hemoglobin in the acid medium (12) and because the reaction between nitrite and hemoglobin proceeds rapidly in such a medium. As seen from the data of Table II, even within these short periods all the nitrite had disappeared when the hemoglobin was in excess and all the hemoglobin had reacted when the nitrite was in excess.

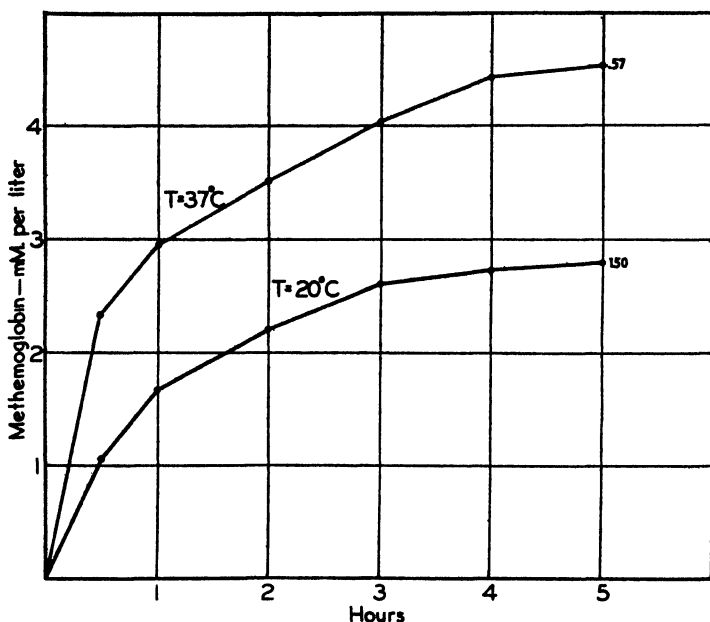


FIG. 2. Methemoglobin and residual nitrite in laked blood after addition of 2.84 mM of sodium nitrite per liter.

Acidification had no influence upon the molar relation of methemoglobin formed to nitrite utilized; the ratios as found ranged from 0.49 to 0.54.

In a final series of experiments, the methemoglobin formed from nitrite was determined in the living animal to test the validity of the findings of von Issekutz (5). He injected sodium nitrite in doses of 8 to 30 mg. per kilo (0.116 to 0.435 mM per kilo) and from the maximum concentration of methemoglobin in the blood, which developed in 30 minutes to 1 hour, he calculated an average nitrite-methemoglobin molar ratio of nearly 1. In the experiments here, rats were given, by intraperitoneal injection, 0.218 and 0.435 mM per kilo of sodium nitrite. At 30 minutes, 1 hour, and each

hour thereafter, to a total of 4 hours, the methemoglobin was determined and, in contrast to the work of von Issekutz (5), the concentration of nitrite. With a dose of 0.218 mm per kilo the methemoglobin concentration reached a maximum of 1.84 mm per liter at 30 minutes; the corresponding nitrite value was 0.218 mm per liter. With a dose of 0.435 mm per kilo the concentration of methemoglobin reached a maximum of 3.85 mm per liter in 1 hour; that of nitrite was then 0.333 mm. In both experiments there were

TABLE I

*Formation of Methemoglobin in Laked Blood As Determined from Nitrite Added and Nitrite Utilized*

Red cells laked with	Moles nitrite added Moles Hb	1 hr, $\frac{\text{moles nitrite}}{\text{moles Hb}}$ calculated from		3 hrs, $\frac{\text{moles nitrite}}{\text{moles Hb}}$ calculated from	
		Nitrite added	Nitrite utilized	Nitrite added	Nitrite utilized
Water	0.5			1.42	0.48
"	1.00			1.62	0.50
"	1.03	2.65	0.50		
Saponin	0.52	2.03	0.48	1.46	0.51
"	1.03	2.65	0.53	1.75	0.54
"	1.05	2.70	0.57	1.78	0.51

TABLE II

*Formation of Methemoglobin in Acidified Blood*

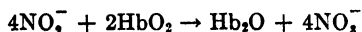
$\frac{\text{Moles nitrite added}}{\text{Moles Hb}}$	MHb formed	Nitrite utilized	Nitrite remaining	$\frac{\text{Moles nitrite utilized}}{\text{Moles MHb}}$
	<i>mm per l.</i>	<i>mm per l.</i>	<i>mm per l.</i>	
0.15	2.11	1.03	0.00	0.49
0.24	2.82	1.46	0.00	0.52
0.30	3.91	2.05	0.00	0.52
0.50	5.46	2.75	0.00	0.50
0.59	6.10	3.23	0.87	0.53
1.16	7.10	3.85	0.35	0.54

still appreciable amounts of nitrite in the blood at the time the maximum amount of methemoglobin was present. In these experiments, and also in those of von Issekutz (5), no allowance was made for the elimination of nitrite in the urine. Thus for two reasons it cannot be assumed that the maximum amount of methemoglobin reached in the blood expresses the reaction of hemoglobin with the total amount of nitrite given. In addition, the maximum amount of methemoglobin observed is the algebraic sum of the amount produced and the amount reduced; this point has already been discussed. The only conclusion justified from such experiments is

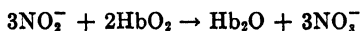
that the molar ratio of nitrite utilized to methemoglobin formed is less than 1.

#### DISCUSSION

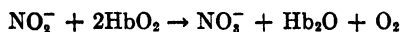
Barcroft and Müller (1) define the relation of methemoglobin formation to nitrite utilization in the statement: "Methemoglobin is formed quantitatively when potassium nitrite is added to blood, the amount of hemoglobin converted containing an amount of dissociable oxygen equivalent to that necessary to convert the nitrite to nitrate." Since each molecule of hemoglobin contains 1 molecule of dissociable oxygen for each atom of iron, it follows from this statement that 2 molecules of nitrite react with 1 molecule of hemoglobin to form 1 molecule of methemoglobin. From present knowledge of methemoglobin as a ferric compound (13) expressed by the formula  $\text{Hb}_2\text{O}$  (or  $\text{HbOH}$ ), it is impossible to write an equation fulfilling the conditions in the statement of Barcroft and Müller (1). Thus the equation



cannot be balanced. In any possible reaction between nitrite and hemoglobin to form methemoglobin and nitrate the amount of hemoglobin converted must always contain an amount of dissociable oxygen greater than that necessary to convert the nitrite to nitrate.<sup>2</sup> One may thus write the equation



The nitrite-methemoglobin molar ratio in this reaction is 1.5. Other equations may be written in which free oxygen is liberated as a product of the reaction,



or



In the first of these equations, the nitrite-methemoglobin molar ratio is 0.5; in the second, it is 1.0.

In the experimental work of Van Slyke and Vollmund (3) from which

<sup>2</sup> Since 2 molecules of methemoglobin are formed by each molecule of nitrite, 0.5 molecule of oxygen should be liberated per molecule of hemoglobin reacting, if the nitrite goes only to nitrate. We have found in four determinations, using no excess of nitrite, a liberation of 0.80, 0.77, 0.79, and 0.80 mole of oxygen per mole of hemoglobin. The determinations were made by the micro gasometric method of Roughton and Scholander (14). These data would indicate that products other than nitrate are produced in the reaction.

they reported a nitrite-methemoglobin molar ratio of 1, laked blood was employed. To 5 cc. portions containing 7.30 mm of hemoglobin per liter there were added 0.05, 0.1, 0.2, and 0.4 cc. of a 1.0 per cent solution of sodium nitrite. These investigators calculated these amounts as representing 0.1, 0.2, 0.4, and 0.8 mole of nitrite per mole of hemoglobin. An arithmetical error appears in this calculation; the amounts of nitrite used are correctly 0.2, 0.4, 0.8, and 1.6 moles per mole of hemoglobin—twice the amounts they state. This recalculation results in a corresponding change in the nitrite-methemoglobin molar ratio from 1, which they report, to 2.

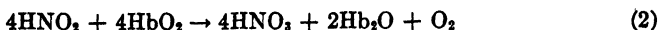
The explanation for this high ratio obtained after correction of the calculations of Van Slyke and Vollmund may be in the facts that the determinations of methemoglobin were made at the end of 30 minutes at room temperature and that none was made for residual nitrite. From a preliminary experiment in which they found virtually all of the hemoglobin converted to methemoglobin in 20 minutes at room temperature, they concluded that 30 minutes were adequate for the reaction. This conclusion would be justified were it known that all of the nitrite added had been utilized. If, as shown here, 1 mole of nitrite reacts with 2 moles of hemoglobin, nitrite was present in considerable excess in their experiment. The rate of reaction of nitrite and hemoglobin depends upon their relative concentrations (15). Thus with an excess of nitrite its relative concentration would become greater as the hemoglobin disappeared and the reaction would proceed at an undiminishing rate. If, however, the hemoglobin were in excess, the relative concentration of nitrite would become progressively smaller with a correspondingly diminishing rate of reaction. The results of the present investigation have, in fact, shown that except in acidified hemoglobin solutions, or when the nitrite is in excess, the reaction is far from complete after 30 minutes, particularly at room temperature. This is in agreement with the observations of Austin and Drabkin (6) who found that upon addition of small amounts of nitrite to hemoglobin solutions the maximum formation of methemoglobin did not occur for many hours.

Meier (4), in estimating a nitrite-methemoglobin ratio of 1, made no determinations of methemoglobin but measured the amount of free oxygen liberated in the reaction. When he added large excesses of nitrite to acid blood, oxygen was taken up. This he explained by the oxidation of nitrite to nitrate which he showed would occur in the presence of acid, in air, without hemoglobin.



At a concentration of nitrite, however, which was just sufficient to convert all of the hemoglobin to methemoglobin Meier found that an amount of

oxygen was liberated equal to one-fourth of the amount in oxyhemoglobin. For this reaction, he therefore wrote the equation



in which 1 mole of nitrite forms 1 mole of methemoglobin. The error made by Meier was that he arbitrarily chose 1 mole of nitrite per mole of hemoglobin as the amount just sufficient to convert all of the hemoglobin to methemoglobin. Had he used 0.5 mole of nitrite per mole of hemoglobin, he would have found that this too was enough to convert all of the hemoglobin. The equation for this reaction is



in which one-half of the oxygen in the oxyhemoglobin is liberated instead of one-fourth of it, as found by Meier. Since he added twice as much nitrite as is necessary, only half of it reacted with the hemoglobin according to Equation 3; the other half reacted with oxygen according to Equation 1, utilizing half of the oxygen liberated in the formation of methemoglobin and resulting in a sum of reactions which is expressed by Equation 2. Meier gave no evidence that all of the nitrite he added reacted exclusively with hemoglobin.

The determination of the nitrite-methemoglobin ratio for the living animal by the method used by von Issekutz (5) is, as pointed out, impossible, since (a) the maximum concentration of methemoglobin occurs at a time when there is considerable residual nitrite, (b) before the maximum is reached, there is reduction of methemoglobin, (c) part of the nitrite is excreted in the urine (16).

Since the nitrite-methemoglobin ratio *in vitro* does not appear to be dependent upon temperature, concentration, or pH and appears to be constant for the reaction between nitrite and hemoglobin, it is probably the same in the living animal as *in vitro*.

#### SUMMARY

1. In the reaction between nitrite and hemoglobin, *in vitro*, 1 molecule of nitrite reacts with 2 molecules of hemoglobin to form 2 molecules of methemoglobin.

2. Temperature, concentration of nitrite, and pH are without influence upon the ratio of the nitrite utilized to methemoglobin formed.

3. In an acid medium the reaction of nitrite with hemoglobin is complete in a short time.

4. In a neutral or slightly alkaline medium, the reaction may take many hours for completion, depending upon the concentration of nitrite.

5. The amount of methemoglobin formed per mole of nitrite utilized

cannot be determined directly in the living animal but is probably the same as it is *in vitro*.

Acknowledgment is made of the technical assistance of Evelyn Shukovsky.

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